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Prodrug Strategies in Anticancer Chemotherapy

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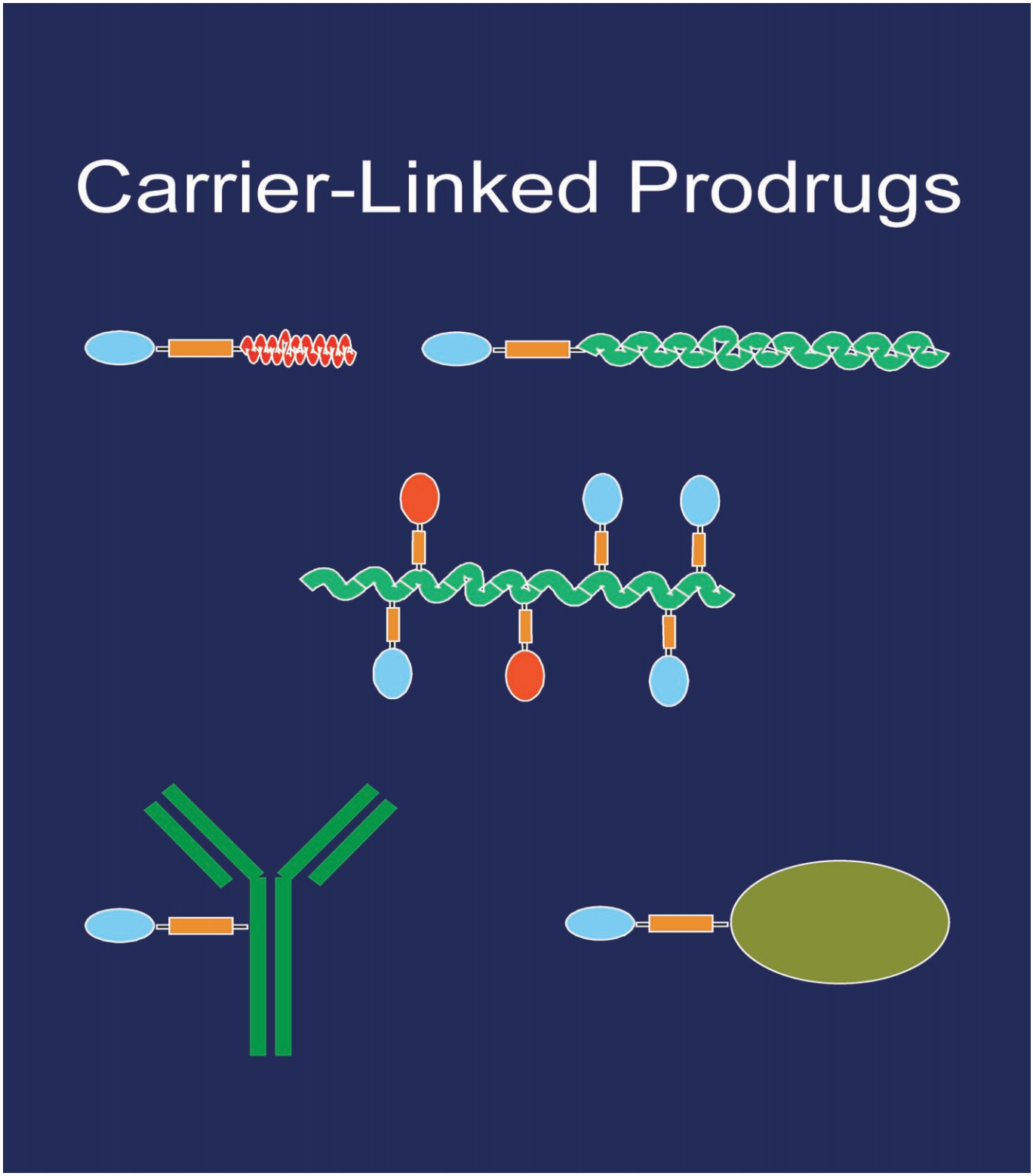

The majority of clinically approved anticancer drugs are characterized by a narrow therapeutic window that results mainly from a high systemic toxicity of the drugs in combination with an evident lack of tumor selectivity. Besides the development of suitable galenic formulations such as liposomes or micelles, several promising prodrug approaches have been followed in the last decades with the aim of improving chemotherapy. In this review we elucidate the two main concepts that underlie the design of most anticancer prodrugs: drug targeting and controlled release of the drug at the tumor site. Consequently, active and passive targeting using tumor-specific ligands or macromolecular carriers are discussed as well as release strategies that are based on tumor-specific characteristics such as low pH or the expression of tumor-associated enzymes. Furthermore, other strategies such as ADEPT (antibody-directed enzyme prodrug therapy) and the design of self-eliminating structures are introduced. Chemical realization of prodrug approaches is illustrated by drug candidates that have or may have clinical importance.

1. Introduction

Prodrugs by definition are derivatives of drugs that are metabolized or activated in the body to release or generate the active drug—if possible at the site of action. Taking this literally, a wide spectrum of clinically established anticancer agents can be considered prodrugs, such as alkylating agents, platinum complexes, antimetabolites, as well as mitomycin C and related derivatives. This is a view that clinicians might not immediately share because the mainstay of modern prodrug development relies on attaching chemical groups or carriers to the drug that are cleaved to release the drug in a tumor cell or tissue. A classic and clinically successful example of such a prodrug is capecitabine (Xeloda, Figure 1), which is activated in a three-step process: First, hepatic carboxylesterase converts it into 5′-deoxy-5-fluorocytidine; second, cytidine deaminase in the liver or tumor yields 5′-deoxy-5-fluorouridine; finally, the tumor-associated enzyme thymidine phosphorylase gives 5-fluoruracil, which in turn is converted into 5-fluorouridine or 5-fluoro-2-deoxyuridine, which are incorporated into RNA and DNA, respectively.[1] Capecitabine is an example of an enzyme-activated prodrug that does not incorporate a carrier molecule. There are further examples of such prodrugs under preclinical or clinical development, such as Tegafur, but these are not the focus of this Review, and we refer the reader to recent comprehensive review articles on this topic.[2,3] The emphasis of this Review is the design of carrier-linked prodrugs that transport the drug to the tumor in a first step and release the drug outside or inside the tumor cell in a second step.

Drug delivery in oncology is of particular interest owing to the narrow therapeutic window of anti-neoplastic agents. In the past, numerous research efforts have concentrated on conjugating anticancer drugs with a wide spectrum of low- and high-molecular-weight carriers including sugars, growth factors, vitamins, peptides, antibodies, polysaccharides, lectins, serum proteins, and synthetic polymers. The general design of carrier-linked prodrugs is shown in Figure 2 with some examples of various carriers. In most prodrug systems the drug is bound to the carrier through a spacer that incorporates a pre-

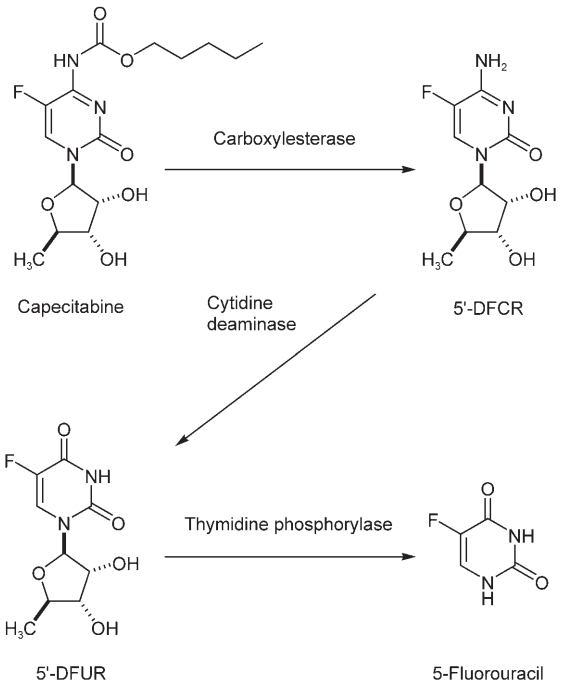

Figure 1. Structure of the 5-fluorouracil prodrug capecitabine (Xeloda), a prominent example of an enzyme-activated prodrug that is converted by three enzymes to 5-fluorouracil.

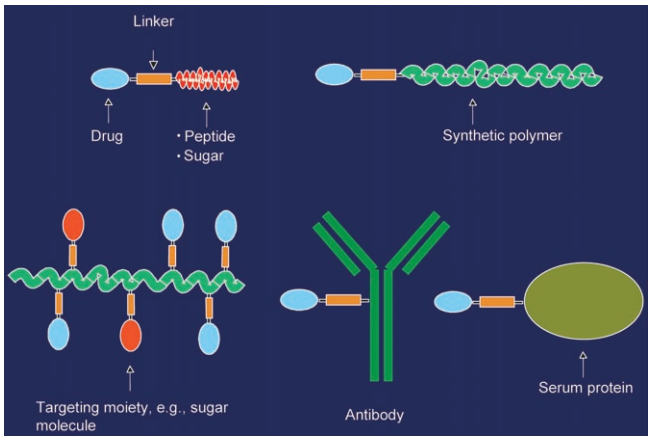

Figure 2. Examples of carrier-linked prodrugs: low-molecular-weight prodrugs generally contain peptides or sugar molecules as the targeting ligand; macromolecular prodrugs make use of synthetic polymers, antibodies, or serum proteins as the drug carrier.

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determined breaking point that allows the bound drug to be released at the cellular target site.

Designing truly tumor-specific carriers remains a challenge in modern drug development. The molecular weight, three-dimensional structure, and immunogenic potential as well as the heterogeneity of the tumor with respect to tumor marker expression, vascularization, and interstitial pressure influence the biodistribution of the drug carrier and dictate the amount of drug that reaches the target site. Optimizing the physicochemical properties of a given carrier is the first critical aspect of carrier-linked prodrug design.

The second aspect relevant for the design of carrier-linked prodrugs is that the modification of the carrier with the drug should preserve the targeting properties of the carrier and ensure a controlled release of the drug inside or outside the tumor cells. The predetermined breaking point introduced in the prodrugs should have sufficient stability in the bloodstream, yet allow the drug to be released effectively at the tumor site by enzymatic cleavage, reduction, or in a pH-dependent manner.

In the following sections we describe fundamental principles of drug targeting and drug release. Subsequent sections are devoted to representative examples of prodrugs that illustrate the salient features of the given targeting strategy and that have reached an advanced stage of preclinical testing or are under evaluation in clinical trials.

2. Prodrug Strategies

2.1. Targeting prodrugs to solid tumors

Carrier-linked prodrug strategies are based on active or passive targeting. Active targeting relies on the interaction of the carrier-linked prodrug with a tumor-associated cell surface marker such as a receptor or antigen. Differences in the biochemical and physiological characteristics of healthy and malignant tissue are responsible for the passive tumor accumulation of macromolecules. Both targeting principles are described below.

2.1.1. Active targeting

Active targeting is based on differences in cell surface antigen or receptor expression between normal and cancer tissue. The aim of active targeting is to develop drug conjugates with monoclonal antibodies (mAbs) or receptor-affine ligands that interact specifically with their cellular target. A suitable carrier combines optimal loading and release properties, long-term circulation, low toxicity, and high affinity for the receptor or antigen without increasing drug levels in healthy tissue.[4,5] Selected cellular targets that have been used for active targeting in cancer therapy are shown in Table 1.

Cell surface targets can be classified as internalizing and non-internalizing systems. In non-internalizing systems the drug conjugate has to be cleaved extracellularly, whereas in in-

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Ivonne A. Müller received her diploma in chemistry from the Julius-Maximilians-Universität, Würzburg (Germany) in 2006. In October 2006 she joined the group of Dr. Felix Kratz at the Tumor Biology Center in Freiburg. She is currently working on her doctoral thesis on the development of acid-sensitive macromolecular anticancer prodrugs.

Dr. André Warnecke studied chemistry at the universities of Clausthal and Freiburg (Germany). In 1997 he received his diploma in the field of metallocene chemistry. Under the supervision of Professor R. Mülhaupt, he conducted his PhD thesis research on albumin-binding prodrugs of anticancer agents which he finished in 2001. He is currently developing suitable chemical architectures for innovative drug-release strategies in the macromolecular prodrug group.

Table 1. Examples of membrane-associated targets for prodrug therapy.

	Receptors	Antigens
Vascular receptors	Integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$) Nucleolin Aminopeptidase N Endoglin Vascular endothelial growth factor receptor (VEGF1–4)	Cluster of differentiation (e.g. CD20, CD33) Carcinoembryonic antigen Blood group carbohydrates Mucin-type glycoproteins (MUC1, CanAg) Lewis Y, Lewis X Cancertestis antigens (CT7, MAGE-A3) Prostate-specific membrane antigen
Plasma protein receptors	Low-density lipoprotein receptor Transferrin receptor	
Peptide receptors	Somatostatin receptor Bombesin receptor Neuropeptide Y receptors Luteinizing-hormone-releasing-hormone receptor	
Receptors for growth factors and vitamins	Folate receptors (FR-α, FR-β, FR-γ) Epidermal growth factor receptors (e.g. EGF1, EGF2, Her2) Transforming growth factor receptor Fibroblast growth factor receptors	
Carbohydrate receptors	Asialoglycoprotein receptor Galectins (e.g. galectin 1, galectin 3) Selectins (e.g. E-selectin, P-selectin) Hyaluronic acid receptors (CD44, RHAMM, HARLEC)	

ternalizing systems cleavage should occur intracellularly after endocytosis.

Historically, antibodies were the first carrier systems that were intensively investigated in active targeting approaches due to a high binding affinity for their respective antigens, and their progress has been extensively reviewed.[6–17] The development of murine mAbs by Milstein and Köhler in 1975[18,19] was an important impetus for cancer research, yielding an array of defined antibodies, each with its own binding specificity for certain tumor-associated antigens.

Monoclonal antibodies are used as single agents, as drug–antibody conjugates, or as antibody–enzyme conjugates (Figure 3). Most antibodies belong to immunoglobulins of the IgG class, which is the smallest but most abundant antibody found in all body fluids. The IgG molecule is a symmetric Y-shaped glycoprotein with two light and two heavy chains joined together by disulfide bridges (Figure 4). The heavy chains are glycosylated, and both chains include a constant and a variable region with high-affinity binding sites for the antigens.[20,21] Antibodies can be cleaved by proteases into defined fragments: pepsin cleaves the antibody to the bivalent $F(ab)_2$ fragment, whereas cleavage by papain produces two monovalent Fab fragments. The smallest unit with complete monovalent binding affinity of an intact mAb is the scFv molecule, a single-chain antibody (Figure 4). Monoclonal antibodies elicit antitumor effects through various pathways: by simple blockage of antigens and subsequent inhibition of signal transduction, by complement-dependent cytolysis (CDC), or by antibody-dependent cell-mediated cytotoxicity (ADCC) (Figure 3).[22] After binding of the antibody to an antigen, the complement cascade is activated and/or effector cells bind to the Fc regions of the antibody, activating natural killer cells and leading to the destruction of the antigenic cell.

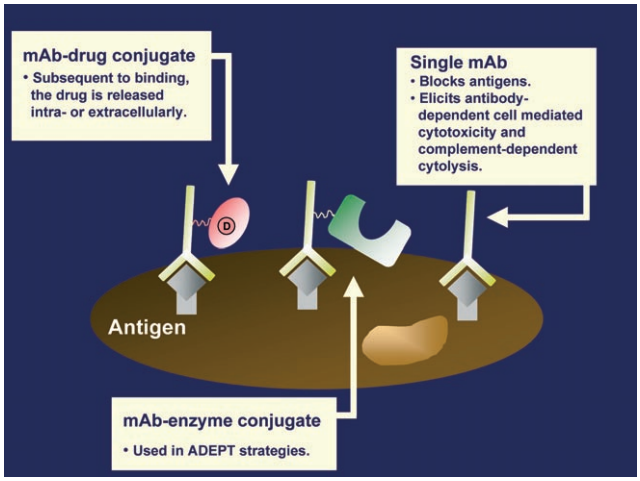


Figure 3. Various uses of mAbs in cancer therapy.

The initial clinical trials with mAbs were disappointing primarily because antibodies of murine origin were used that provoked immune reactions in the treated patients. The advent of chimeric, humanized, or human mAbs, in which only the variable, hypervariable, or none of the regions of the binding domain carry murine sequences, have, in principle, resolved this drawback. As a consequence, five antibodies, trastuzumab

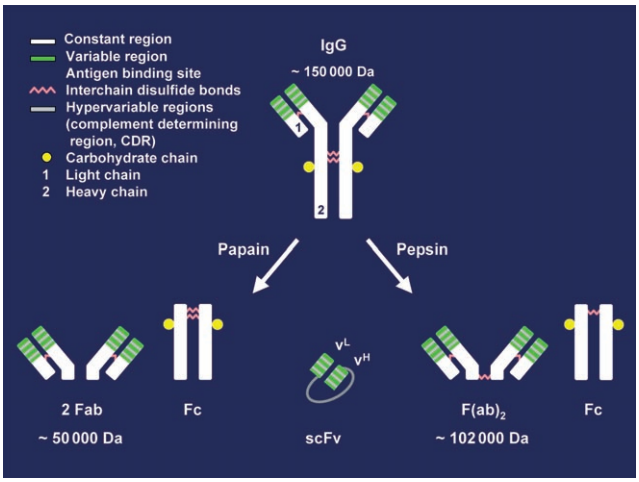


Figure 4. Schematic presentation of immunoglobulins IgG and their fragments Fab, Fc, scFv, and F(ab)$_2$.

(Herceptin), alemtuzumab (Campath), rituximab (Rituxan), bevacizumab (Avastin), and cetuximab (Erbitux) have been approved by the U.S. Food and Drug Administration (FDA) for treating hematological and solid cancers,[23–25] and a large number of other antibodies are in clinical trials.

In contrast, mAb conjugates with clinically established agents have been disappointing.[10,12,26–28] The main reason for this lack of clinical success is probably that a sufficient amount of drug has to be transported to the tumor; on the other hand, unconjugated antibodies already exert immune-modulated cytotoxicity when small amounts of the mAbs bind to the respective antigen. As a consequence, modern drug–antibody conjugates use highly cytotoxic drugs, toxins, or radionuclides.[12,24,27,29,30] Selected examples are given in Table 2.

Table 2. Selected drug–antibody conjugates and immunotoxins in cancer therapy.

Drug	Conjugate	Antigen/Receptor	mAb/Cytokine	Ref.
Calicheamicin	Mylotarg	CD33	P67.6	[192, 199]
Maytansinoid DM1	Cantuzumab Mertansine	CanAg	huC242	[208]
Auristatin E	SGN-35	CD30	cAC10	[221]
Yttrium-90	Zevalin	CD20	Ibritumomab	[284]
Iodine-131	Bexxar	CD20	Tositumomab	[285]
Mutated diphtheria toxin	TransMID 107	Transferrin receptor	Transferrin	[156, 157]
Mutated *Pseudomonas* exotoxin	IL13-PEI-301-R03	IL-13 receptor	Interleukin 13	[156, 157]

Besides antigens, cellular receptors provide further targets for carrier-linked prodrug design (Table 1). There are many tumor-associated receptors that have been investigated in receptor-targeting approaches, such as the transferrin (a circulatory iron carrier protein) receptor,[31] selectins,[32] integrins,[33] the folate receptor, GLUTs (glucose uptake transporters),[34–36] galectins (with high affinity for β-galactosides),[37] hyaluronic acid receptors,[38,39] and the asialoglycoprotein receptor (ASGPR).[39,40]

Active targeting is accomplished by binding drugs to suitable ligands that display a high affinity for the individual receptor. The ligand-based prodrug subsequently binds to the receptor and is taken up by receptor-mediated endocytosis. The drug is then released in the endosomes and/or lysosomes depending on the route of cellular trafficking of the receptor in question. Suitable ligands can be low- or high-molecular-weight compounds; typical candidates are vitamins,[41] peptides,[42] sugars,[43] native or modified proteins,[44] and antibodies.[23–25]

Folic acid, one of the most popular ligands, retains high affinity for its receptor,[45–47] even when linked to a variety of molecules. The folate receptor is overexpressed in various human cancers,[48–52] and a broad spectrum of low- and high-molecular-weight drug–folate conjugates with alkylating agents, platinum complexes, paclitaxel, 5-fluorouracil, camptothecin, doxorubicin, and mitomycin has been investigated.[53–58]

Promising results for vascular receptor targeting were obtained with cyclic peptides that bind to integrins. These receptor proteins are crucial for the interaction between a cell and the extracellular matrix and are particularly involved in tumor angiogenesis. Certain integrins (mainly $\alpha_v\beta_3$ and $\alpha_v\beta_5$) are overexpressed on proliferating endothelial cells and some tumor cells.[59] Angiogenesis can be inhibited by targeting integrins with peptides containing an RGD sequence (Arg-Gly-Asp) that is present in extracellular matrix proteins.[60] In subsequent work, a number of RGD–drug conjugates with cytostatic and diagnostic agents[61–64] were developed, and a proof of concept has been obtained for several candidates (reviewed in references [65–69]).

The ASGPR, a membrane-bound lectin expressed on hepatocytes and liver cancer, is another receptor that has been targeted using prodrugs with the goal of improving the treatment of hepatocellular carcinoma.[70,71] This receptor has a high affinity for terminal β-galactoside and β-*N*-acetylgalactosamine residues on glycoproteins[72,73] and is responsible for the endocytosis of several glycoproteins.[40,74,75] The strong interaction of glycoproteins with the ASGPR is due to the "cluster-glycoside effect"[76] in which adjacent saccharide groups (multivalent ligands or so-called glycoside clusters) are responsible for high binding constants. This effect is mainly attributed to thermodynamic properties of multivalent ligands (comparable to the chelate effect) rather than to the presence of multiple receptor binding sites. Various carriers,[77,78] drug conjugates,[79–84] and imaging agents[85] with partly remarkable binding constants for the ASGPR have been investigated during the past years.

Conceptually, receptor and antigen targeting is an attractive strategy for tumor-selective delivery of drugs, ideally propagating the therapeutic concept of drug targeting founded on Paul Ehrlich's vision of "the magic bullet" that he proclaimed at the

beginning of the 20th century. However, there are several reasons that prevent effective delivery of prodrugs that are based on active targeting principles:

a) Receptor-affine ligands and antibodies are not exclusively tumor-specific,[86,87] and cross-reactivity of the drug conjugates with normal tissue can cause systemic toxicity.
b) The inner regions of solid tumors are often poorly vascularized and exhibit relatively low blood flow. Both factors decrease the amount of the macromolecular prodrug reaching these parts of the tumor.[88]
c) Heterogeneity of antigen and receptor expression by tumor cells restricts the number of cells that can be targeted effectively. Tumor cells that express the antigen or receptor at low levels or not at all will most likely escape therapy.
d) To some extent, antigens and receptors are secreted into the circulation.[89] The "shedding" of the antigen or receptor from the surface of tumor cells limits the amount of drug reaching the tumor because soluble antigens and receptors neutralize the prodrugs in the circulation.
e) Preclinical evaluation of prodrugs mostly relies on mouse models with human transplanted tumors. The biodistribution of prodrugs with mAbs and ligands in these models might not, however, be indicative of the biodistribution behavior in humans.[90]

To overcome some of these problems, the sequential application of nontoxic prodrugs and enzyme conjugates has been pursued in more complex prodrug strategies such as ADEPT (antibody-directed enzyme prodrug therapy).

ADEPT denotes a two step-mechanism that was developed independently by Bagshawe[91] and Senter.[92] Initially, a tumor-associated mAb linked to a drug-activating enzyme is administered intravenously (Figure 5). This conjugate binds to a specific antigen expressed on the surface of the tumor cell. After a suitable time, a low-molecular-weight prodrug is administered and converted by the enzyme into a cytotoxic drug. The enzyme needs to activate the prodrug with high selectivity and turnover at physiological conditions without inducing a strong immune response. Moreover, it should not be present in normal tissue to prevent activation of the prodrug outside the tumor cell. Table 3 lists selected enzymes and drugs that have been used in ADEPT.[93–98]

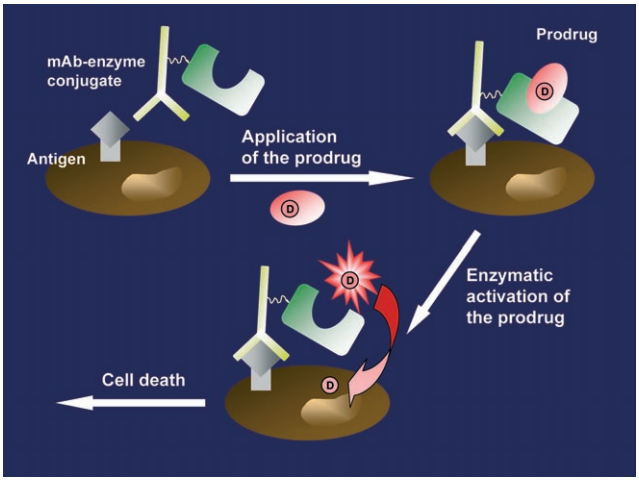


Figure 5. Schematic presentation of antibody-directed enzyme prodrug therapy (ADEPT).

Table 3. Selected enzymes and drugs for ADEPT.

Enzyme	Drug	Ref.
Carboxypeptidase G2	Benzoic acid mustard	[286]
	Doxorubicin	[287]
Carboxypeptidase A	Methotrexate	[288, 289]
Aminopeptidase	Methotrexate	[290]
β-Glucuronidase	Doxorubicin	[291]
	Camptothecin	[292]
	5-Fluorouracil	[293]
β-Lactamase	Nitrogen mustards	[294]
	Doxorubicin	[295]
	Paclitaxel	[296]
Catalytic antibodies	Camptothecin	[297]

Although numerous preclinical studies have validated ADEPT concepts, clinical development is restricted to a few phase I studies. Early trials were carried out with A5CP, a conjugate of the bacterial enzyme carboxypeptidase G2 (CPG2) with the $F(ab)_2$ fragments of an anti-CEA mAb, and a benzoic acid or bis-iodophenol mustard prodrug.[99–101] The observed slow enzyme elimination required an additional antibody clearance step before prodrug administration, and the immune response to A5CP did not allow repeated administrations. An advanced system, MFECP1, a fusion protein of CPG2 and the single-chain scFv antibody MFE-33, was investigated in phase I/II studies and showed a rapid clearance of the bis-iodophenol mustard prodrug from the circulation, sufficient tumor localization, and a lower immunogenic potential than previous enzyme complexes.[102, 103] In general, stable disease was observed in these studies.

2.1.2. Passive targeting

Active targeting of tumor-specific cell receptors or antigens proceeds at the cellular level. Another more universal strategy exploits anomalies of malignant tissue on a vasculolymphatic level that directly result from the tumor's pathophysiology. Following this approach, often classified as passive targeting, an accumulation of drugs in tumor tissue is simply achieved by employing large molecules (synthetic or biopolymers) or nanoparticles (liposomes, nanospheres) as inert carriers that do not necessarily interact with tumor cells but strongly influence the drug's biodistribution. The underlying concept has been termed enhanced permeability and retention (EPR) and was elucidated by Maeda and co-workers in the mid-1980s.[104] This section gives a short overview of the basic principles, the scope and limitations of the EPR effect as a prerequisite for passively targeting solid tumors, and the requirements for polymers used as macromolecular carriers for anticancer drugs.

For more in-depth information, we refer the reader to a number of recently published reviews on mechanisms of drug transport to solid tumors including the EPR effect.[88,105–107]

At a size of 2–3 mm, tumor cell clusters induce angiogenesis to satisfy their increasing demands for nutrition and oxygen. The new blood vessels formed during this process often differ markedly from those of normal tissue. Neovasculature generated by the tumor is characterized by an irregular shape and dilated, leaky or defective vessels. The endothelial cells are poorly aligned or disorganized with large fenestrations (Figure 6). Other differences affect the perivascular cells, the basement membrane, and the smooth-muscle layer which are frequently absent or abnormal.[105]

These anatomical features make the vasculature of tumor tissue permeable to macromolecules or even larger nanometer-scale particles such as liposomes and polymeric micelles, whereas in the blood vessels of healthy tissue only small molecules can pass the endothelial barrier. The pore size of tumor microvessels was reported to vary from 100 to 1200 nm in diameter (depending on the anatomic location of the tumor).[108,109] In contrast, the tight junctions between endothelial cells of microvessels in most normal tissues are less than 2 nm in diameter [notable exceptions include postcapillary venules (up to 6 nm) and the kidneys, liver, and spleen (up to 150 nm)].[110] Macromolecules used as carriers for the development of macromolecular prodrugs typically have hydrodynamic radii that are >2 nm and <10 nm (e.g. serum albumin (67 kDa) has an effective diameter of 7.2 nm), allowing extravasation into tumor tissue but not into normal tissue.

However, the enhanced uptake of macromolecules in tumor tissue cannot be solely explained by an enhanced permeability of the vascular system, as this would affect smaller molecules in a similar manner. A more striking difference between small and large molecules is found in the decreased clearance from the tumor if the molecular weight exceeds 40 kDa.[111] Whereas smaller molecules were shown to be rapidly cleared from the tumor interstitium, large molecules are retained, thus showing high intratumor concentrations even after 100 h post-application.[111] This enhanced retention of macromolecules in tumor tissue is primarily caused by a lack of lymphatic drainage due to an impaired or absent lymphatic system. Hence, it is the combination of both enhanced permeability and retention (EPR) that is responsible for the accumulation of macromolecules in solid tumors, as illustrated in Figure 7.

Today, a number of factors are known to influence the EPR effect. Primarily, the extent of accumulating macromolecules depends on the size and type of the tumor. Large tumors that usually have extensive avascular regions are less EPR active than smaller tumors. However, because neovasculature is a prerequisite for EPR, macromolecular prodrugs are not capable of targeting small metastases at a pre-angiogenic stage. Furthermore, it has been found that transplanted tumors (e.g. in xenograft models) are often better vascularized than spontaneously growing tumors.[110] Other factors reported to have a positive impact on tumor vascular permeability are high blood pressure and certain vascular mediators such as bradykinin, nitric oxide, prostaglandins, matrix metalloproteinases, and peroxynitrite.[105,112]

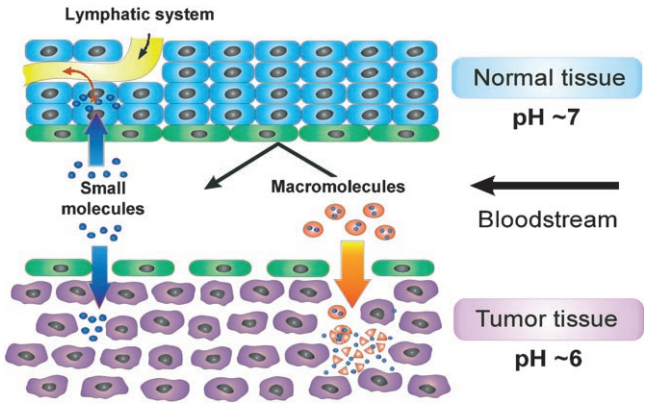


Figure 7. Schematic representation of the anatomical and physiological characteristics of normal and tumor tissue with respect to vascular permeability and retention of small and large molecules (EPR effect).

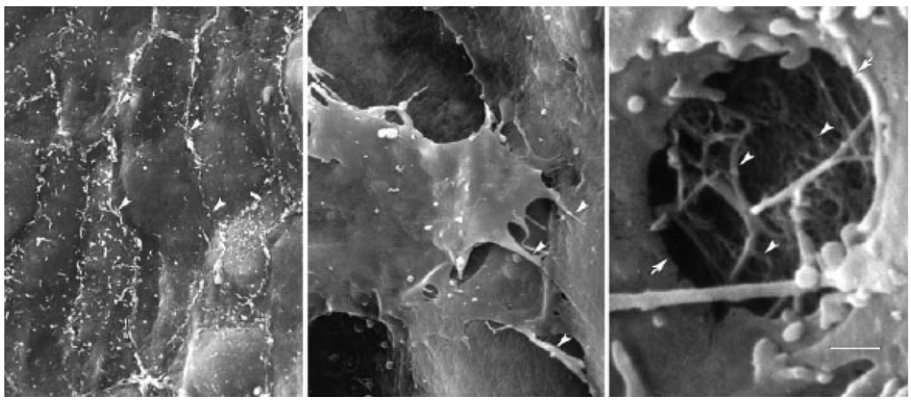


Figure 6. Scanning electron micrographs of the luminal surface of healthy (mouse mammary gland, left) and tumor (MCa-IV mouse mammary carcinoma, center and right) blood vessels. Whereas the healthy vessel is smooth and has tight endothelial junctions, the tumor vessel shows widened intercellular spaces, overlapping endothelial cells, and other abnormalities. A high-magnification view of a hole in the endothelium (right image) makes the underlying basement membrane visible. Scale bar in the picture at right applies to all panels: left, 5 μm; center, 2 μm; right, 0.5 μm. Reprinted from reference [374] with kind permission from D. M. McDonald (University of California, San Francisco), P. L. Choyke (National Institutes of Health, Bethesda), and the Nature Publishing Group, copyright 2003.

Besides the EPR effect, macromolecular prodrugs show one further important difference relative to low-molecular-weight drugs: Because macromolecules are not efficiently cleared by the kidneys, they show an enhanced circulatory retention with prolonged plasma half-lives. Al-

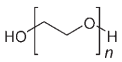
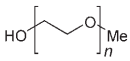
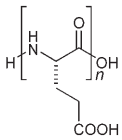
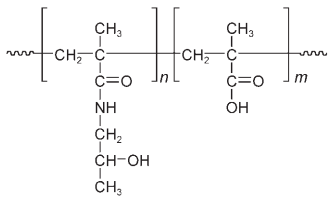
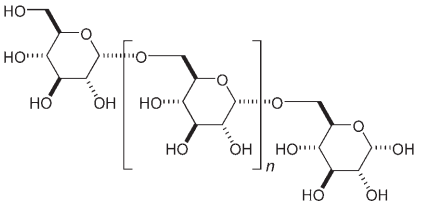
though the glomerular permeability of a molecule strongly depends on various factors such as molecular size, shape, charge, and deformability,[113] a rule of thumb can be applied to most macromolecular carriers: uncharged or negatively charged molecules larger than 40 kDa efficiently escape renal clearance.[113,114] This is often formulated as a prerequisite for a synthetic polymer acting as a macromolecular carrier. Moreover, the appropriate carrier molecule has to fulfill further requirements: it should be sufficiently water soluble, nontoxic, nonimmunogenic and should ideally be biodegradable and have low polydispersity. Table 4 gives an overview of various synthetic polymers that have been frequently used as macromolecular carriers. Other important carrier molecules for the development of macromolecular prodrugs are serum proteins such as transferrin (78 kDa) or human serum albumin (HSA, 67 kDa),[44] the latter of which can be selectively loaded with drugs in situ to form 1:1 conjugates.[115]

For most of the macromolecular carriers described above, a significant accumulation in tumor tissue was shown in biodistribution studies performed in tumor-bearing rodents with the respective radioactively or fluorogenically labeled macromolecule.[111,116–120] Typical intratumor concentrations of the labeled macromolecules that were reached within 50 h after administration were in the range of 1–5% of the injected dose per gram of tumor, but could also be as high as 20% g^{-1}.[118,120] In some of the experiments, the influence of the molecular weight was also studied. Effective long-term accumulation in tumor tissue was observed for PEG with M_w ~200 kDa,[117] HPMA copolymers with M_w 40–800 kDa,[111] and dextran with M_w 40–70 kDa.[116] Although often referred to as such, the molecular weight of a macromolecule is not the appropriate universal parameter for predicting the molecule's biodistribution. Other factors such as the chemical nature of the polymer as well as its shape and conformation in aqueous solution (e.g. globular, linear, or branched) also have a strong impact on the size of the macromolecule, or more precisely, its hydrodynamic radius.[121,122] For instance, Gillies et al. showed that the circulation times of polyester dendrimer–PEG hybrids increased with the degree of branching.[123] In contrast, the charge of the polymer does not seem to have a strong influence on tumor uptake.[124]

A recent extension of the basic passive targeting strategy is polymer-directed enzyme prodrug therapy (PDEPT).[125,126] Conceptually similar to ADEPT (Section 2.1.1), PDEPT is a two-step antitumor approach combining two polymer conjugates: an enzymatically cleavable macromolecular prodrug and the respective polymer–enzyme conjugate. Passive accumulation of both components should enable a controlled release of the cytotoxic drug at the tumor site. In contrast to ADEPT, PDEPT proposes initial administration of the polymeric drug to promote tumor targeting before administration of the activating polymer–enzyme conjugate (Figure 8). This could have two potential advantages over the ADEPT approach:

1) The relatively short plasma residence time of the polymeric prodrug allows subsequent administration of the polymer–enzyme without fear of prodrug activation in the circulation.

Table 4. Examples of frequently used polymeric drug carriers.

Name	Structure	Maximal drug loading
Poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO)		2
Methoxypoly(ethylene glycol) (mPEG)		1
Poly(glutamic acid) (PG)		*n*
N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers		*m*
Dextran		3*n*

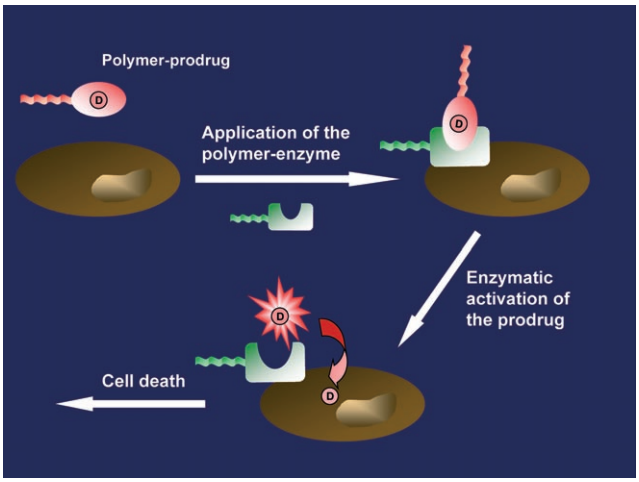


Figure 8. Schematic presentation of polymer-directed enzyme prodrug therapy (PDEPT).

2) Conjugating exogenous enzymes with synthetic polymers could decrease their immunogenicity (e.g. PEGylation). Initial preclinical evidence for this prodrug concept has been obtained with HPMA–enzyme conjugates and HPMA conjugates of doxorubicin.[125,126]

Biodistribution experiments that support the EPR concept by demonstrating an enhanced tumor uptake of macromolecules have usually been carried out in animals, mostly rodent xenograft models. Less information is available from studies using orthotopic tumor models, and little is known about the EPR activity of native tumors in humans. However, in two recent studies, the degree of tumor accumulation of two clinically assessed macromolecular prodrugs was determined in patients who underwent elective surgery after intravenous administration of the macromolecular prodrug. Sarapa et al. investigated the tissue uptake of MAG–CPT (see Figure 22), an 18-kDa HPMA copolymer conjugate of the topoisomerase I inhibitor camptothecin, in patients with colorectal carcinomas.[127] Interestingly, neither a preferential uptake nor an enhanced retention of the conjugate in tumor tissue was observed in comparison with adjacent healthy tissue. Similar results were achieved with DE-310 (see Figure 22), a 340-kDa carboxymethyldextran conjugate of the camptothecin analogue DX-8951, in six patients with different tumors.[128]

Although these results are in marked contrast to previous biodistribution studies in tumor-bearing animals,[129,130] they should not be overestimated. The molecular weight of MAG–CPT is certainly too low for a HPMA copolymer conjugate to show long-term accumulation.[111] Moreover, statistically significant results would have required a greater number of patients. More such studies are needed and would be of great value because they can provide information about the EPR effect in the clinical setting. In addition, prescreening of patients with appropriate diagnostic agents for evaluating the EPR effect of the primary tumor and metastases will be an important asset before initiating clinical trials with drug–polymer conjugates.

2.2. Controlled release at the tumor site

Once inside the tumor, the carrier-linked prodrugs must be cleaved to exert their antitumor efficacy. Cleavage to the free drug can occur extra- or intracellularly. In the past, researchers initially focused on developing tailor-made cleavable spacers that exploit the endosomal/lysosomal pathway of prodrugs; more recent efforts have concentrated on the extracellular cleavage of prodrugs that is mediated through the activity of proteases, for example, by those secreted by the tumor cells.

Low- and high-molecular-weight prodrugs that contain a suitable ligand are taken up by the tumor cell through receptor-mediated endocytosis, and macromolecular prodrugs that follow a passive targeting approach are taken up by adsorptive or fluid-phase endocytosis.[88,131,132] As depicted in Figure 9, invaginations occur at the cell surface during endocytosis, and endosomes are formed which migrate into the cytoplasm. Depending on the macromolecule and the kind of endocytosis process involved, a series of sorting steps take place in which the endosome is either transported to certain cell organelles (such as the Golgi apparatus), returns to the cell surface (recycling), or forms primary and secondary lysosomes.[132] The pH decrease during endocytosis is considerable: from pH 7.2–7.4 in the extracellular space, to pH 6.5–5.0 in the endosomes, and

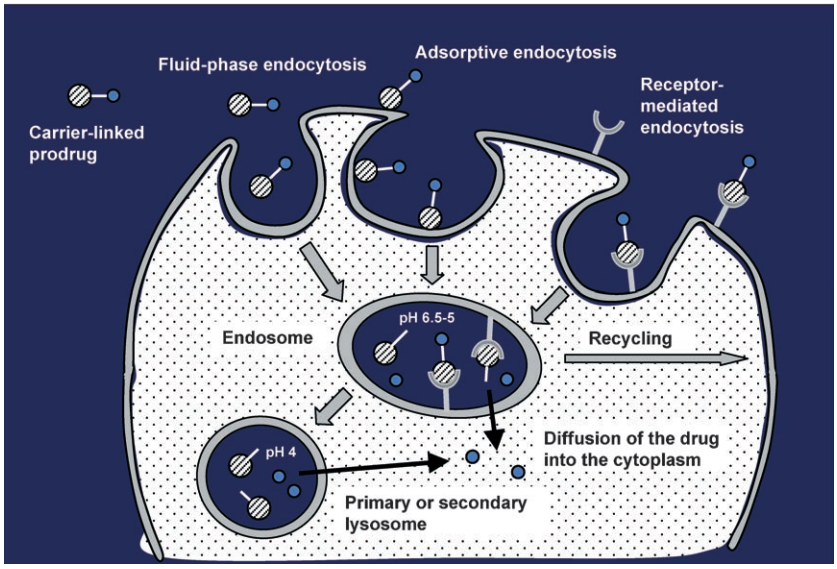


Figure 9. Cellular uptake of macromolecular prodrugs by fluid-phase, adsorptive, or receptor-mediated endocytosis.

to around pH 4.5–4.0 in primary and secondary lysosomes. In the lysosomes a great number of enzymes such as esterases, proteases, and lipases become active.

2.2.1. Acid-promoted liberation of drugs

The acidic conditions in tumor tissue, endosomes, and lysosomes can be responsible for the cleavage of acid-sensitive prodrugs. Based on the pioneering work of Otto Warburg, who described cancer cells that convert glucose to lactate, even in non-hypoxic conditions, which lowers the pH value of the tumor environment,[133] noninvasive techniques with pH electrodes have demonstrated that the environment in tumor tissue is often 0.5–1.0 pH units lower than in normal tissue.[134] This pH shift, although small, could contribute to the extracellular release of acid-sensitive prodrugs, especially if the prodrug remains in the tumor interstitium for longer periods of time.

A larger pH shift from 7.2–7.4 in the blood or extracellular spaces to 4.0–6.5 in the various intracellular compartments takes place during cellular uptake of the carrier-linked prodrugs. The significant drop in pH value is a unique physical property in living systems that can be exploited for intracellular drug delivery by coupling drugs to suitable carriers through acid-sensitive bonds.

When designing carrier-linked prodrugs with acid-sensitive spacers, the functional groups of the anticancer agent and the linker determine the synthetic possibilities. Typical examples of acid-sensitive bonds are shown in Figure 10. By far, most of the synthetic work with macromolecular prodrugs has concentrated on the carboxylic hydrazone bond, followed by the *cis*-aconityl bond and the trityl bond.[135] In addition, a number of low-molecular-weight prodrugs with alkylating properties have also been realized that incorporate acid-labile acetal bonds.[136]

The pH-dependent stability of carrier-linked prodrugs has to fit into a narrow window of 2–3 pH units to become therapeutically relevant. The realization of suitable linkers is not trivial: Not only does the nature of the chosen chemical bond and its substituents affect the pH-dependent stability of the prodrug but also the molecular weight of the carrier as well as the site of attachment on the carrier.[135] In addition, acid lability can be markedly influenced by the type and ionic strength of the buffer used in subsequent hydrolysis studies.[135]

Besides the possibility of attaching drugs to the carrier through acid-cleavable bonds, another interesting strategy was pursued by developing polymeric carriers that incorporate pH-sensitive bonds in their backbone. Recently, a number of different linear polymers were published in which the monomer units were linked by ketal, acetal, and *cis*-aconityl bonds (Table 5).[137–141] These model compounds were designed to undergo a breakdown of the polymer backbone under the acidic conditions after cellular uptake and thus have the benefit of being biodegradable. It was assumed that controlled degradability can prevent the polymer from vacuolization and facilitate endosomal escape of the released drug through membrane destabilization by large numbers of low-molecular-weight polymer fragments.[137] When comparing the half-lives of the published prototype polymers at neutral and acidic pH as depicted in Table 5, one of the poly(amidoamine)s synthesized by the Fréchet research group displayed promising degradation kinetics (that is, sufficient stability at pH 7.4 and relatively fast degradation at pH 5). However, the M_w value of 6.5 kDa is far too low for passive targeting, and the molecule does not provide functional groups for attaching drugs. Apparently, this innovative approach still requires further optimization to produce acid-sensitive biodegradable polymers suitable for an application as carriers for anticancer drugs.

2.2.2. Hypoxia-mediated release/immunotoxins

Because some regions in tumor tissue are poorly vascularized, hypoxic cells are formed that show characteristically low oxygen tension, low pH, low nutrient levels, and overexpression of angiogenic factors. These hypoxic cells are often resistant to radiation and chemotherapy, and thus there is a need for developing active agents that selectively target hypoxic areas. Such bioreductive prodrugs can be activated either by the reducing environment or by bioreductive enzymes that are often expressed at high levels in tumor tissue. Bioreductive enzymes are one- or two-electron reducing systems such as DT-diaphorase or cytochrome *c* (P450) reduc-

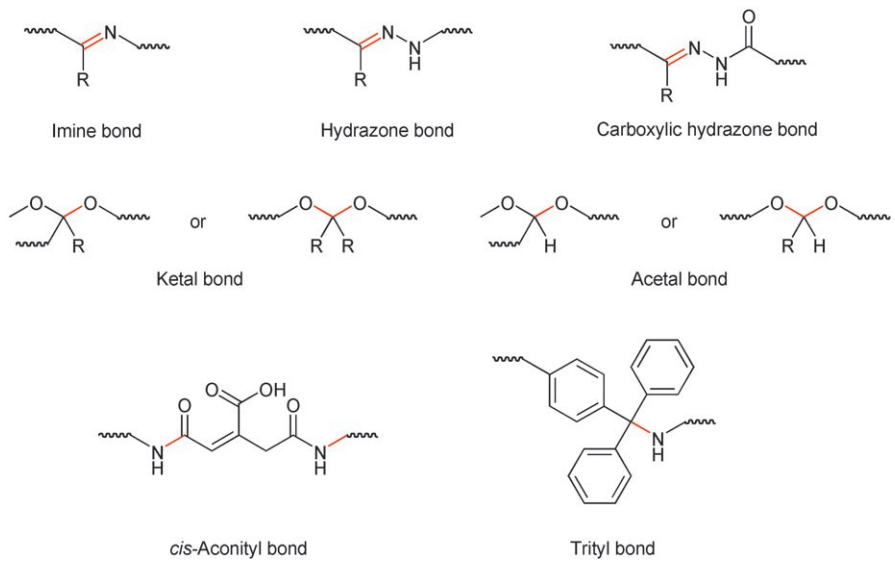
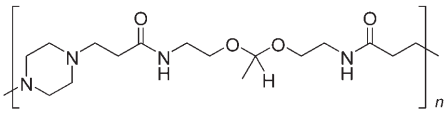
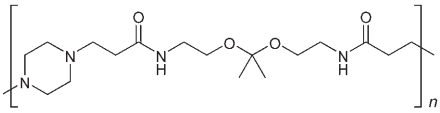
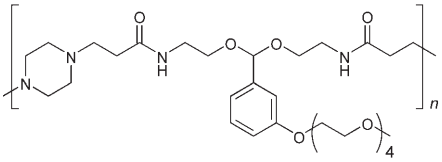
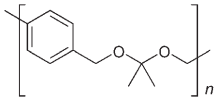
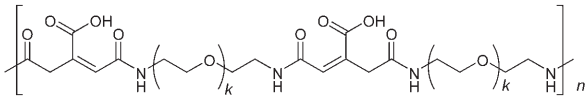
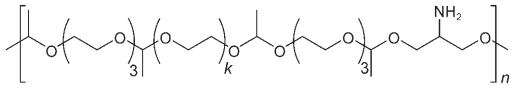


Figure 10. Examples of acid-sensitive bonds that are used for realizing acid-labile prodrugs (the cleavable bonds are highlighted red).

Table 5. Polymers designed as potential pH-sensitive biodegradable drug carriers.

Ref.	Acid-sensitive bond	Structure	M_n [kDa]	M_w [kDa]	$t_{1/2}$ pH 5.0	$t_{1/2}$ pH 7.4
[137]	Acetal		9.8	18.8	81 days	161 days
[137]	Ketal		3.3	6.5	0.03 days	6 days
[137]	Acetal		6.4	13.5	3 days	15 days
[138]	Ketal		2.6	4.0	35 h	102 h
[139]	*cis*-Aconityl		NA[a]	61	>6 days[b]	>6 days
[140]	Acetal		12.5	25	24 h[c]	>21 days[d]

[a] NA: not available. [b] At pH 5.5. [c] ~40% loss of M_w at pH 5.5. [d] ~30% loss of M_w after 21 days.

tase.[142–144] Prodrug design has concentrated on the development of low-molecular-weight prodrugs that are based on three different reductive systems: the quinones, the nitroaromatics/heterocycles, and the *N*-oxides.[142,144] A great number of these prodrugs that are reduced to the active drug under hypoxic conditions have been or are in clinical trials.[145–151]

There are several carrier-linked prodrugs that are activated by a reducing environment in the endosomes/lysosomes, usually occurring by a thiol exchange reaction of a disulfide-containing linker. This reduction can be the sole release mechanism for the prodrug or can take place concomitantly with pH-dependent or enzyme-mediated cleavage of an additional predetermined breaking point introduced in the prodrug. Examples are found in antibody conjugates with highly potent drugs (see Section 3) and immunotoxins.[152–154] Immunotoxins contain a toxin made by plants, insects, or microorganisms, for example *Pseudomonas* exotoxin A (PE), diphtheria toxin (DT), or ricin that comprise three domains (a binding domain (B), an activity domain (A), and a translocation domain (T)). First-generation immunotoxins were constructed by chemical conjugation of the whole toxin with a mAb by a disulfide bond (Figure 11). The presence of the binding domain turned out to be problematic, as it was found that healthy cells were also affected by the toxin. Hence, second-generation immunotoxins were developed in which the binding domain was absent or mutated (Figure 11). In this way, binding of the immunotoxin to healthy cells was suppressed and side effects were decreased. Second-generation immunotoxins with a bacterial toxin often incorporate a thioether bond instead of a disulfide bridge, as the former has enhanced plasma stability. To minimize production costs, third-generation or recombinant

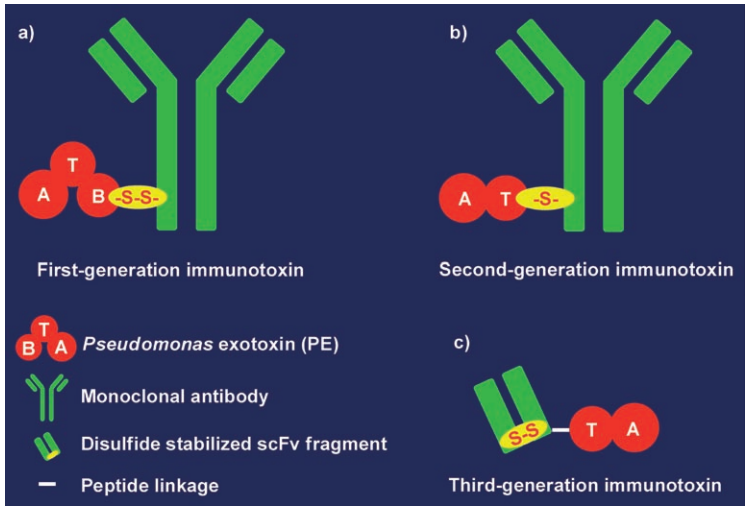


Figure 11. a) First-, b) second-, and c) third-generation immunotoxins of *Pseudomonas* exotoxin (PE) (A: activity domain, B: binding domain, T: translocation domain).

immunotoxins were developed (Figure 11). They are prepared by recombinant DNA techniques and contain only the scFv fragments, which are attached to the toxin by a peptide linkage and not by a disulfide bond.[152–154]

After binding to a cancer cell, immunotoxins are taken up by endocytosis. Activation of the immunotoxins is then realized in two steps: enzymatic cleavage and reduction within the T-domain of the toxin. Owing to the various and very complex mechanisms that cause cell death, the cellular processes that lead to apoptosis are exemplified by an immunotoxin with PE: After endocytosis, the T chain of the toxin is cleaved in the acidic compartment by the lysosomal protease furin, which produces two peptide fragments that are still bound covalently by a disulfide bridge. In a next step, the disulfide is reduced in the lysosomes to liberate a C-terminal subdomain of the translocation domain that is now able to translocate to the cytosol. In the cytosol the A chain of the toxin ADP-ribosylates elongation factor 2, thus inhibiting protein synthesis and inducing apoptosis.[154] In contrast to common anti-neoplastic agents, a single toxin molecule is effective enough to kill one cancer cell.[154,155]

Approximately 30 immunotoxins were evaluated in clinical trials in the 1990s showing efficacy primarily against leukemia but not solid tumors.[154] The focus of newly developed immunotoxins is the treatment of glioblastoma, the most common brain tumor in adults. Due to the systemic toxicity of immunotoxins, they are administered through catheters directly into the brain tumor. Promising candidates in clinical trials are the immunotoxins TransMID 107 (transferrin–CRM107) and PRECISE (IL13-PEI-301-R03). TransMID 107 is an immunotoxin of transferrin and CRM 107, a mutated diphtheria toxin. In a phase II trial with 33 patients this immunotoxin was administered to patients with glioblastoma multiform or anaplastic astrocytoma, showing five complete remissions and seven partial remissions;[156] phase III trials are ongoing. The immunotoxin IL13-PEI-301-R03, which consists of a recombinant mutated *Pseudomonas* exotoxin combined with the cytokine IL-13 is also being evaluated against glioblastoma in a phase III trial at present.[156,157]

2.2.3. Release of drugs through enzymatic cleavage

Activation of a prodrug can be realized by tumor-associated enzymes such as proteases, glucuronidase, or carboxylesterases, which are expressed either intra- or extracellularly by normal and malignant cells. Probably the best known examples of intracellular proteases that degrade proteins in lysosomes are cathepsins, especially cathepsin B. The proteases that are extracellularly expressed, such as the matrix metalloproteases, are responsible for the proteolysis of the extracellular matrix and basement membranes that is required during embryo morphogenesis, tissue remodeling, angiogenesis, and parasitic or bacterial invasion.[158–162] Malignant cells use proteolysis in a similar manner to normal cells, but they combine it with motility. This difference leads to invasion and metastasis. Attachment of tumor cells to the basement membrane and their detachment are necessary steps involved in the formation of metastases during which malignant cells produce degradative enzymes in high concentrations. This overexpression together with an imbalance between proteases and their inhibitors characterize the invasive and metastatic potential of the individual tumor.

There are two different approaches for exploiting tumor-associated enzymes: the enzymes can be inhibited[163–168] or their activity can be used for controlled release of a carrier-linked prodrug at the tumor site. In the past, a great number of carrier-linked prodrugs have been developed with enzyme-specific substrates such as peptides or sugars. An overview of enzymes overexpressed by tumors and examples for respective substrates is given in Table 6.

In principle, three different mechanisms of drug release from enzymatically cleavable prodrugs are conceivable that are illustrated by the doxorubicin prodrugs shown in Figure 12. In the three doxorubicin prodrugs:

a) A peptide linker is bound directly to the 3′-amino sugar of the drug and can be cleaved by an enzyme at this position to release doxorubicin.[169]
b) Cleavage occurs within the peptide sequence with subsequent liberation of a drug–peptide derivative that is further cleaved or hydrolyzes to the active compound.[170]
c) The peptide linker is attached to the drug by a self-immolative spacer (Section 2.2.4) and cleaved by an enzyme that produces a labile self-immolative spacer–drug derivative that in turn eliminates spontaneously to release the anticancer agent.[171]

Because the enzymes that are used for prodrug activation are also present in normal cells, activation of an enzymatically cleavable prodrug can be observed in healthy tissue, too. This stresses the need to improve tumor uptake through active or passive targeting and to characterize the overexpression of the respective enzymes in the individual tumor, preclinically as well as clinically.

2.2.4. The double prodrug approach

The double prodrug approach generally refers to the use of pro-prodrugs (prodrugs of prodrugs) and has proven advantageous over single prodrug strategies in many cases (e.g. capecitabine, Figure 1).[172] In the field of carrier-linked anticancer prodrugs, however, this strategy has been primarily restricted to the use of a certain class of cross-linkers: so-called self-immolative (or self-eliminating) linkers that are capable of improving the release of drugs in an elegant fashion.

Prodrugs designed for enzymatic activation have often proven to be poor substrates for their respective enzymes. These problems are caused by steric hindrance if the bond located adjacent to the (bulky) drug molecule is intended as the cleavage site and does not have access to the active site of the enzyme. To effectively circumvent these drawbacks, double prodrugs were developed that are based on the general principle illustrated in Figure 13. The tumor-specific cleavage reaction takes place between the trigger and the linker to form an

Table 6. Overview of enzymes that are overexpressed in tumors.

Enzyme	Function	Substrate examples	Ref.
Cathepsin B (EC 3.4.22.1) Cathepsin H (EC 3.4.22.16) Cathepsin L (EC 3.4.22.15)	Lysosomal degradation of proteins	Arg-Arg, Leu, Ala-Leu, Gly-Leu-Phe-Gly, Gly-Phe-Leu-Gly, Ala-Leu-Ala-Leu	[298–306]
Cathepsin D (EC 3.4.23.5)	Degradation of extracellular matrix	Phe-Ala-Ala-Phe(NO_2)-Phe-Val-Leu-OM4P, Bz-Arg-Gly-Phe-Phe-Pro-4MβNA	[307]
Plasmin (EC 3.4.21.7)	Fibrinolysis, degradation of blood plasma proteins	D-Val-Leu-Lys, D-Ala-Phe-Lys, D-Ala-Trp-Lys	[177, 308–316]
uPA (EC 3.4.21.73)[a] tPA (EC 3.4.21.68)[b]	Activation of plasmin formation	Gly-Gly-Gly-Arg-Arg Arg-Val	[317] [318]
Prostate-specific antigen [PSA (EC 3.4.21.77)]	Liquefaction of semen	Mu[c]-His-Ser-Ser-Lys-Leu-Gln-Leu, L-377,202[d]	[319–327]
Matrix metalloproteases [MMP-2 (EC 3.4.24.24), MMP-9 (EC 3.4.24.35)]	Degradation of extracellular matrix and collagens	Ac-Pro-Leu-Gly-Leu, Ac-γE-Pro-Cit-Gly-Hof[e]-Tyr-Leu, Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln	[170, 328–331]
β-Glucuronidase (EC 3.2.1.31)	Hydrolysis of glucuronide moieties from proteins	Glucuronide moieties	[171, 332–338]
Carboxylesterases [CES1/CES2 (EC 3.1.1.1)]	Hydrolysis or transesterification of drugs or xenobiotics	Ester or carbamate moieties	[339–344]

[a] Urokinase-type plasminogen activator. [b] Tissue-type plasminogen activator. [c] Mu = morpholinocarbonyl. [d] L-377,202 = *N*-glutaryl-(hydroxypropyl)-Ala-Ser-cyclohexylglycyl-Gln-Ser-Leu. [e] Hof = homophenylalanine.

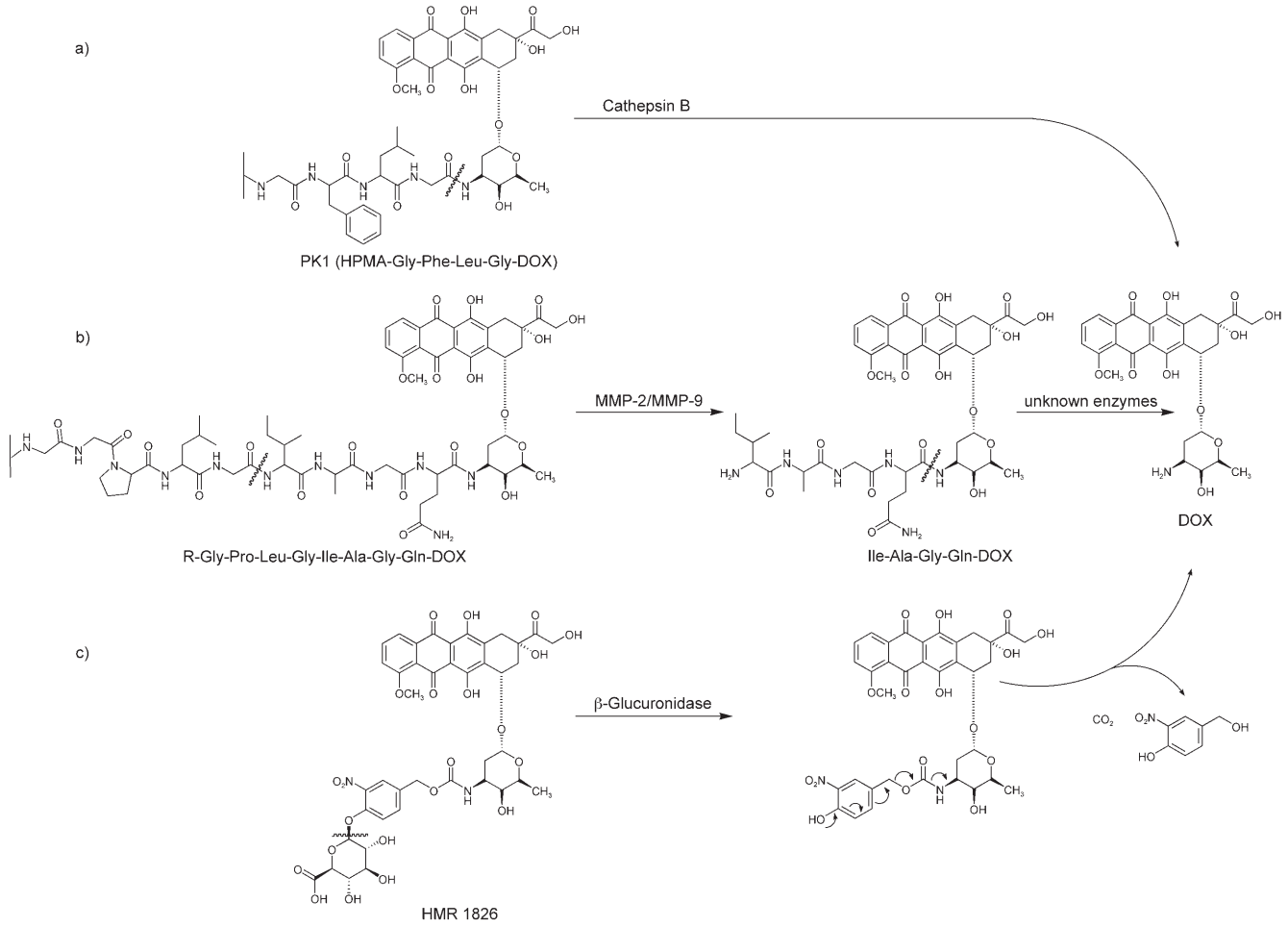


Figure 12. Various drug-release strategies for enzymatically cleavable prodrugs exemplified by prodrugs of doxorubicin (DOX).

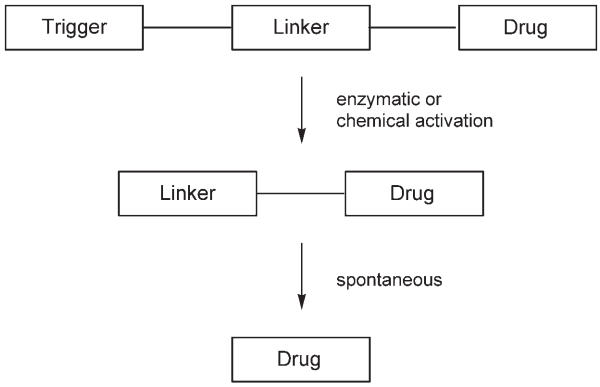


Figure 13. General principle of drug release in double prodrug approaches.

intermediate drug–linker derivative that decomposes spontaneously to liberate the unmodified drug. Over the years, a considerable number of such self-immolative linkers has been developed and used in double prodrug approaches (reviewed in references [173, 174]). According to their mechanism of drug release, the linkers can be classified as cyclization or elimination linkers, with the latter tending to be cleaved more rapidly. Relevant examples of self-immolative linkers including their mechanism of drug release are summarized in Table 7. One of the first and most abundant linkers is the *p*-aminobenzyloxycarbonyl (PABC) system, introduced by Carl et al.,[175] that is cleaved in a 1,6-benzyl elimination reaction. An impressive example of how effective the use of such spacers can be in the design of enzymatically cleavable prodrugs was given by Dubowchik et al., who synthesized a series of doxorubicin derivatives with cathepsin B-cleavable dipeptides (Table 8).[176] In the presence of the target enzyme, only those derivatives that incorporated the self-immolative PABC moiety showed a liberation of the drug, whereas for the other prodrugs in which the

Table 7. Examples of self-immolative linkers and their reaction mechanisms for releasing the parent drug.

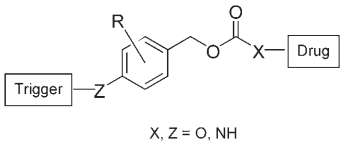
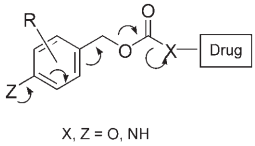
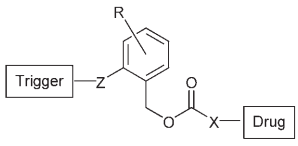
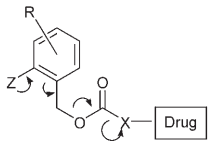
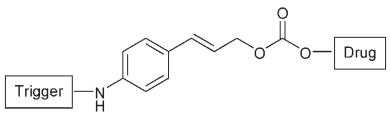
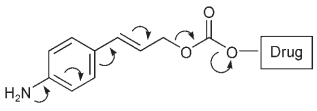
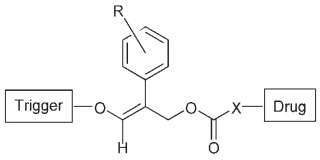
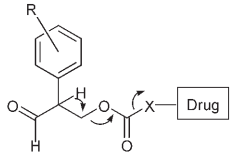
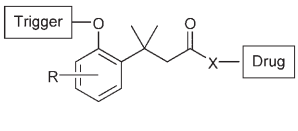
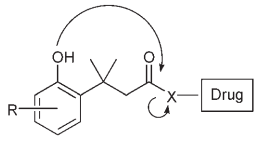
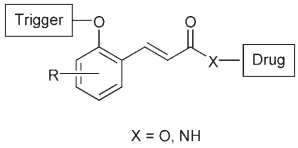
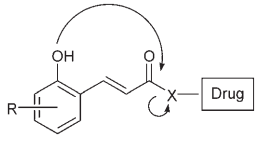
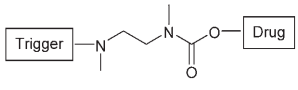
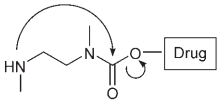
Linker	Ref.	Reaction type	Release mechanism
	[174, 175, 314, 345, 346]	1,6-Benzyl elimination	
	[174, 345]	1,4-Benzyl elimination	
	[347]	1,8-Elimination	
	[348]	β-Elimination	
	[349]	Cyclization (lactonization)	
	[350, 351]	Cyclization (lactonization)	
	[315]	Cyclization	

Table 8. Half-lives of doxorubicin (DOX) release from dipeptide–PABC–DOX[a] and dipeptide–DOX derivatives.[224]

Substrate	$t_{1/2}$[b]
Z[c]-Phe-Lys-DOX	≫ 7 h
Z-Phe-Lys-PABC-DOX	9 min
Z-Val-Cit-DOX	≫ 7 h
Z-Val-Cit-PABC-DOX	4 h

[a] PABC = *p*-aminobenzyloxycarbonyl. [b] A solution of substrate (40 μM) was incubated with activated cathepsin B (40 nM) at 37 °C and pH 5. [c] Z = benzyloxycarbonyl.

peptide was directly coupled to the amino position of doxorubicin, no cleavage was observed during the experiment. Studies by de Groot et al.[177] underscore the importance of further increasing the distance between the predetermined breaking point and the drug for effective cleavage. Peptide derivatives of doxorubicin and paclitaxel that incorporate elongated self-immolative linkers (dimers and trimers of the PABC system) were subjected to plasmin cleavage. Elongation of PABC resulted in an enhanced rate of cleavage relative to homologous compounds containing a single PABC linker.

A recent logically consistent refinement of the self-immolative linker technology is the development of dendrimers that, after a single triggering event, undergo a cascade reaction resulting in the disassembly of the entire dendritic scaffold. The synthesis of such compounds, optionally denoted as "cascade-release" or "self-immolative" dendrimers, was reported independently and almost simultaneously by the research groups of D. Shabat, F. M. de Groot, and D. V. McGrath[178–180] and has already been reviewed.[181,182] Self-immolative dendrimers are based on self-immolative linkers as branching units and can be terminally loaded with drugs. Activation at the focal point initiates a cascade of elimination reactions that consequently lead to the release of a number of drug molecules (schematically depicted in Figure 14). This simultaneous multiple release of drugs upon a single activation step makes these compounds attractive for a use as "intelligent" drug carriers.

Nevertheless, there are some present drawbacks of this concept: The main problem is that only a restricted number of drug molecules fit into the limited space of the outer shell of the dendrimer due to steric hindrance. A third-generation dendron with eight small dye molecules and a second-generation dendron with four bulky paclitaxel molecules are the largest dendrimer drug conjugates that could be synthesized up to now.[178,179]

Despite the large number of self-eliminating dendrimers that have been synthesized in the last years,[183–186] most of them were model compounds and in vivo data are still lacking, probably due to the poor water solubility of the dendrimer–drug conjugates. In a recent innovative approach, Shabat and co-workers[187] attached PEG polymers by click chemistry to the branching units of a self-immolative dendritic prodrug of camptothecin. This dendrimer has improved water solubility, and its degradation mechanism is presented in Figure 15. In vitro data show that the cytotoxicity of the prodrug is increased 100-fold in the presence of the enzyme penicillin G amidase (PGA).

3. Characteristic Examples of Anticancer Prodrugs

In this section, we describe pertinent prodrugs with antibodies, synthetic polymers, and serum proteins as well as prominent examples of prodrugs that target tumor-associated receptors.

Drug conjugates with antibodies

Early clinical trials with drug–antibody conjugates with clinically established agents have had limited success due to a low degree of tumor accumulation combined with insufficient cytotoxicity of the drug used.[13] As a consequence, scientists reasoned that the development of immunoconjugates with cytotoxic agents active in the nanomolar or sub-nanomolar range would be more successful, especially if the level of antigens expressed on cancer cells is low (e.g. CD33).[188] Suitable drugs are DNA-strand-breaking xenobiotics (calicheamicins) or inhibitors of tubulin polymerization (auristatins, maytansinoids). Despite their unacceptable systemic toxicity, these drugs have lower immunogenicity than toxins. Mylotarg,[189–192] a calicheamicin–antibody conjugate (Figure 16), is the first of this new generation of drug–antibody conjugates to be approved and further candidates are under clinical development (Section 4).

Calicheamicin γ_1^I, derived from the organism *Micromonospora echinospora*, is a relatively small molecule with $M_w \sim$ 1500 Da that belongs to the enediyne antibiotics.[193] The structure of calicheamicin consists of three parts (Figure 17): The aryl tetrasaccharide domain (blue) anchors the enediyne moiety sequence-selectively to the minor groove of the DNA strand; subsequently, a thiol (such as glutathione) reduces the trisulfide portion (red), initiating the reaction cascade in which the enediyne moiety (black) forms diradicals via Bergmann cyclization[194] with subsequent hydrogen abstraction from the opposite sugar–phosphate backbone, initiating a conformational change in the DNA by an induced-fit mechanism[195] and subsequent strand scission.[196]

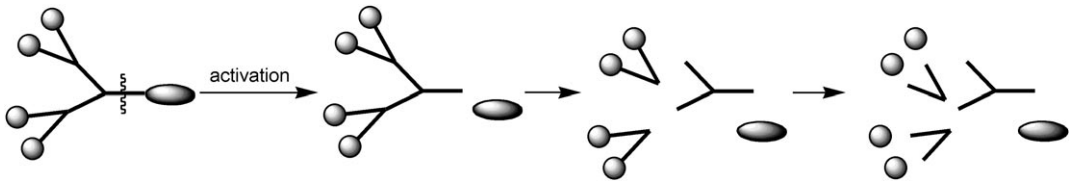


Figure 14. General principle of tail-unit release from a self-eliminating dendrimer after activation at the trigger.

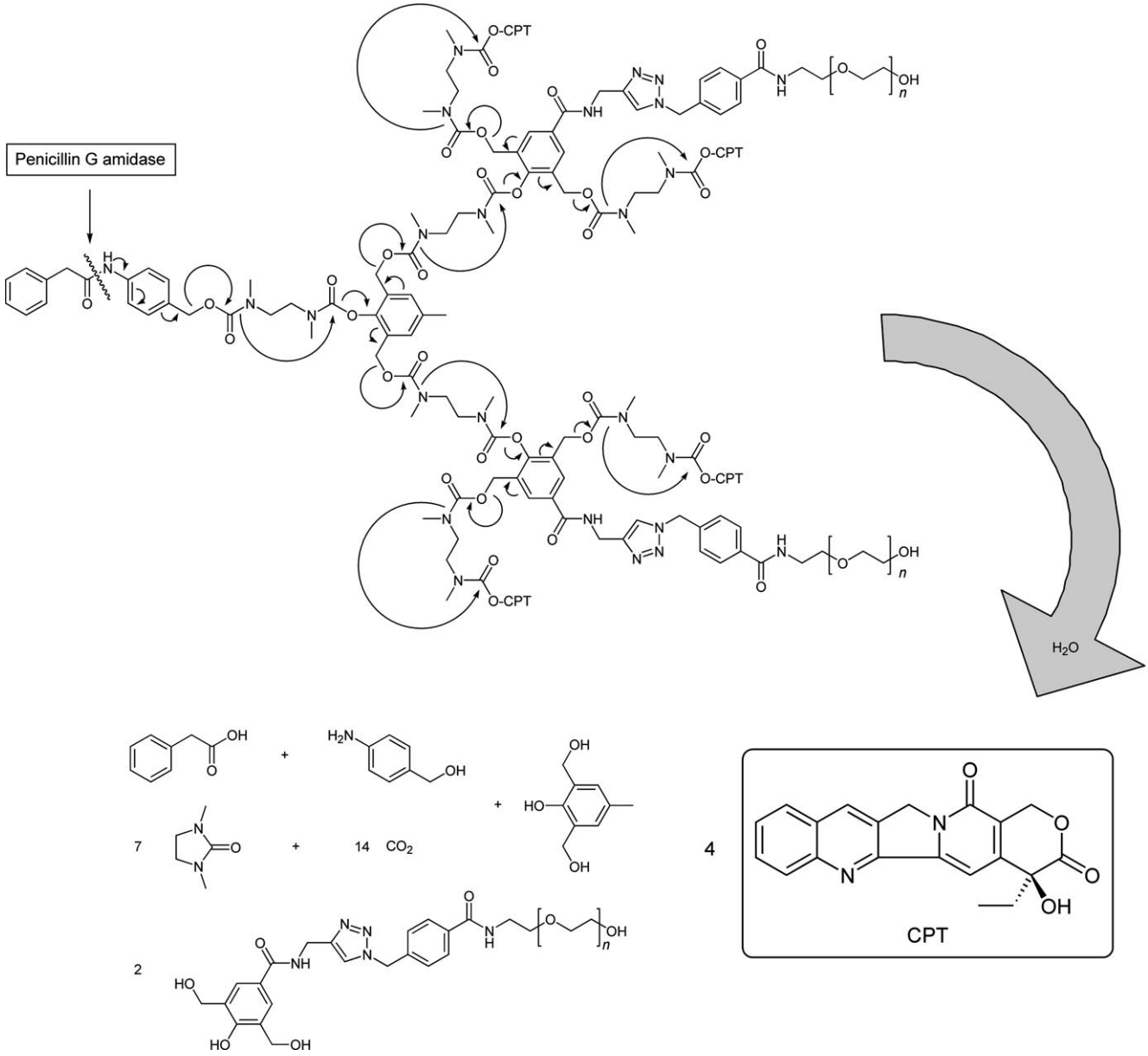


Figure 15. Dendritic prodrug of camptothecin (CPT) that decomposes after cleavage with penicillin G amidase thus releasing four molecules of the drug (and a considerable number of various by-products from the fragmentation of the polymer backbone).[187]

Calicheamicin–antibody conjugates are disulfide versions of the trisulfide parent compounds; two different coupling strategies were investigated: Hydrazide derivatives of calicheamicin, attached to the oxidized carbohydrates on antibodies, form so called "carbohydrate conjugates" that release calicheamicin dimethyl hydrazide (CalichDMH) after hydrolysis in the acidic endosomes/lysosomes and subsequently activate the enediyne by reduction of the disulfide. In contrast, "amide conjugates", which lack the hydrazone bond, are attached to the ε-amino group of lysine residues of the antibody and leave the disulfide part as the sole site for drug release. In both conjugates the disulfide was stabilized by two adjacent methyl groups to enhance serum stability, and the conjugates contained an acetylated amino sugar in the tetrasaccharide moiety, which was found to exert optimal antitumor efficacy in preclinical models.[197] For the mAb CTM01 that targets the MUC1 antigen, the amide conjugate showed superior antitumor efficacy in vivo compared with the carbohydrate conjugate.[198] Surprisingly, subsequent studies with the antibody P67.6 that targets another antigen, CD33, showed the opposite result, because with this drug-delivery system the disulfide was not cleaved effectively and therefore the acid-labile hydrazone bond was essential for selective cytotoxicity.[199] This highlights that an optimal design for one antibody is not necessarily valid for another antibody. Additional introduction of a 4-(4′-acetylphenoxy)butanoic acid (AcBut) spacer leads to the most prominent calicheamicin antibody–conjugate Mylotarg, consisting of CalichDMH and the AcBut spacer which is covalently linked to the

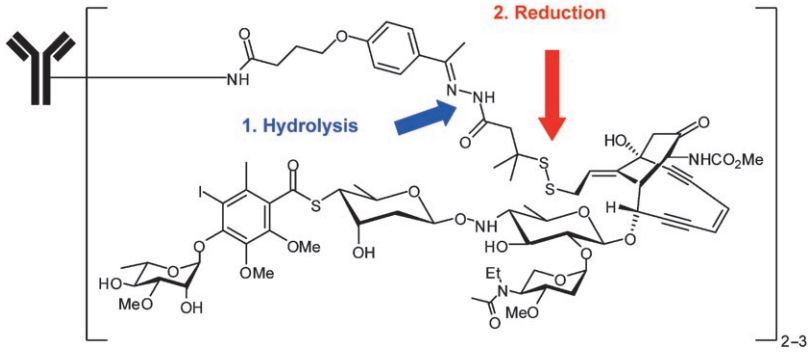


Figure 16. Structure of Mylotarg, consisting of the humanized antibody P67.6, the 4-(4′-acetylphenoxy)butanoic acid (AcBut) linker, and NAc-γ-calicheamicin dimethyl hydrazide (CalichDMH). Following release of the drug by hydrolysis of the hydrazone bond in the acidic endosomes/lysosomes, the enediyne is activated by reduction of the disulfide bond.

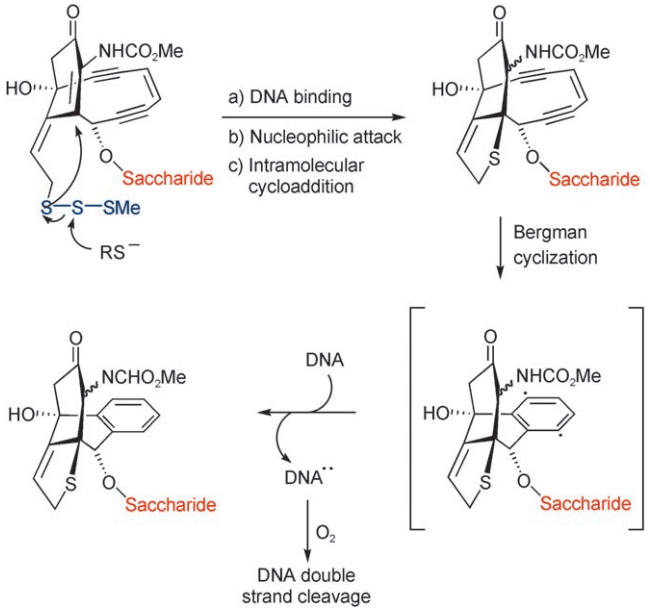


Figure 17. DNA-cleaving mechanism of calicheamicin γ_1^I. After DNA binding, the trisulfide part is reduced by a thiol (e.g. glutathione) followed by intramolecular Michael addition of the thiolate to the adjacent α,β-unsaturated ketone, and elimination of the bridgehead double bond. The enediyne moiety forms a diradical, which abstracts a hydrogen from the DNA resulting in DNA cleavage.

ε-amino group of lysines of the humanized antibody P67.6 (Figure 16).[192]

Another class of highly cytotoxic drugs used for developing potent antibody–drug conjugates includes the microtubule inhibitors.[200] These can be classified into depolymerization inhibitors (e.g. paclitaxel) and the polymerization inhibitors, such as Vinca alkaloids, auristatins, or maytansinoids, the latter two being active in the subnanomolar range. Maytansine, isolated by Kupchan et al. from the Ethiopian shrub *Maytenus serrata* at the National Cancer Institute in 1972,[201] is a 19-membered macrocyclic lactam of an ansamycin antibiotic. Maytansine reversibly binds to assembled tubulin, causing disassembly of microtubules and hence inhibiting mitosis.[202–204] Over the years, other maytansinoids were found in plants and microorganisms with a number of structural variations including different ester chains at the C3-position, the presence or absence of the 4,5-epoxide, the *N*-methyl group, the halogenide, and the C14 methyl group. Nevertheless, the presence but not the nature of the ester chain at C3 is crucial for inhibitory activity.[205–207] Maytansinoids have been coupled to mAbs or natural ligands such as folate.[208–216] All mAb–maytansinoid conjugates are derivatized at the C3 ester chain and contain a disulfide linker. Cantuzumab mertansine (Figure 18), the most advanced disulfide drug conjugate, consists of the maytansinoid DM1, which is coupled to the ε-amino groups of lysine residues of the humanized antibody C242 by a disulfide bond.[208] The most likely mechanism for release of the active drug is reductive cleavage of the disulfide bond in endosomes/lysosomes which ensures an intracellular release of DM1.[211]

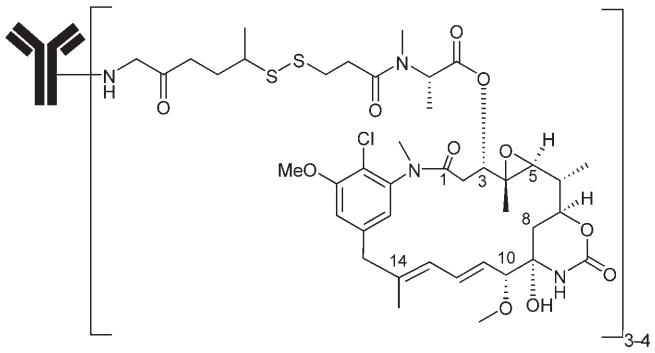


Figure 18. Structure of Cantuzumab mertansine (huC242–DM1), which consists of the maytansinoid DM1 linked by a disulfide bridge to the ε-amino group of the lysine residues of the humanized mAb C242.

A further promising class of drugs for antibody-based cancer therapy are auristatins,[217] which are about 50- to 200-fold more potent than Vinca alkaloids.[29] Auristatins are structural analogues of the marine pentapeptide dolastatin 10, which interacts with the Vinca alkaloid binding site on α-tubulin and inhibits polymerization of tubulin, hence preventing formation of the mitotic apparatus.[218–220] Several auristatin conjugates have been evaluated, such as cAC10–vcMMAE or cBR96–hydrazone-MMAE. The chimeric mAb cAC10, which induces growth arrest of $CD30^+$ cells in vivo and in vitro,[221,222] was covalently linked to monomethyl auristatin E (MMAE) through a valine–citrulline (vc) peptide linker (Figure 19), and selectively cleaved by lysosomal enzymes such as cathepsin B after internalization.[223,224] The acid-labile auristatin conjugate with the chimeric anti-Lewis Y cBR96 mAb showed lower stability in human

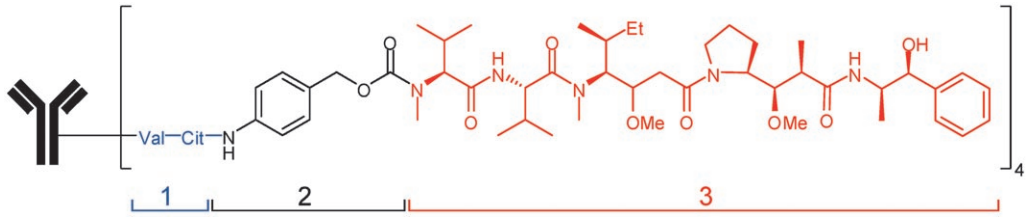


Figure 19. Structure of the conjugate cAC10–vcMMAE, consisting of the cleavable valine–citrulline peptide linker (1), the self-immolative spacer (2), and monomethyl auristatin E (3).

serum than an analogous dipeptide conjugate.[225] cAC10–vcMMAE (SGN-35)[226–228] was chosen as a candidate for clinical evaluation after further preclinical antigen binding studies of the cAC10–vcMMAE conjugate with four and eight drugs per mAb showed the best efficacy for the conjugate with four molecules per mAb.[229] Other antibody conjugates with MMAE targeting CD20,[230] CD70,[231] and the tumor-associated glycoprotein NMB[232] are under preclinical development, as are conjugates with auristatin MMAF, which has lower toxicity, attenuated potency, and improved aqueous solubility relative to MMAE.[233]

Prodrugs with polymeric carriers

In 1975, Ringsdorf and co-workers proposed a general schematic design of a drug-delivery system using synthetic polymers for low-molecular-weight drugs.[234,235] One to several drug molecules are bound to a polymeric backbone through a spacer that incorporates a predetermined breaking point to ensure release of the drug after cellular uptake of the conjugate (Figure 20). The system can also contain solubilizing

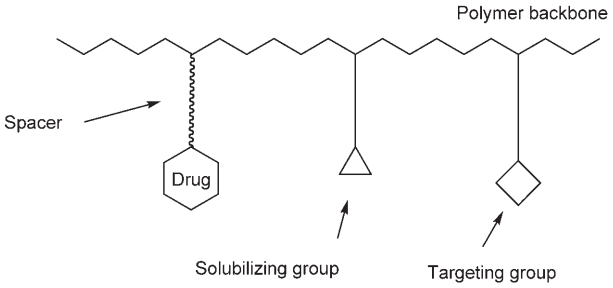


Figure 20. Ringsdorf's model for a polymeric drug containing the drug, solubilizing groups, and targeting groups bound to a linear polymer backbone.

groups or targeting moieties which render the conjugate biorecognizable. Inspired by this pioneering work, numerous anticancer drug–polymer conjugates with various macromolecular carriers have been developed over the last three decades. The vast majority of them are logically consistent applications of Ringsdorf's model.

In contrast to drug conjugates with antibodies, drug–polymer conjugates that rely on EPR-mediated accumulation have been realized with clinically established anticancer drugs such as doxorubicin, methotrexate, camptothecin, paclitaxel, and platinum analogues. Research in this field has concentrated mainly on *N*-(2-hydroxypropyl)methacrylamide (HPMA)-based copolymers, poly(ethylene glycol) (PEG), and poly(glutamic acid) (PG) as water-soluble drug-delivery vehicles, but there are also some recent developments worth mentioning that have focused on more sophisticated biodegradable drug carriers, such as dendrimers and PEG–dendrimer hybrids. In addition, serum albumin, an abundant endogenous protein, is under evaluation as a drug-delivery system in anticancer therapy. Below, we describe four drug–polymer conjugates: PK1, a HPMA copolymer conjugate with doxorubicin; PEG–CPT (Prothecan or Pegamotecan), a PEG conjugate with camptothecin; Xyotax, a PG conjugate with paclitaxel; and an acid-sensitive doxorubicin conjugate with a dendritic polymer that characterize the salient features of drug conjugates with synthetic polymers.

PK1 was the first macromolecular prodrug to enter clinical trials. Manufactured by radical copolymerization of *N*-(2-hydroxypropyl)methacrylamide and *N*-methacryloylglycylphenylalanylleucylglycine *p*-nitrophenylester and subsequent reaction of the *p*-nitrophenyl-activated polymeric precursor with doxorubicin, PK1 has a molecular mass of approximately 28 kDa with ~8.5 wt% of doxorubicin linked to the polymer (Figure 21).[236] The development of PK1 followed the biological rationale that cellular uptake of the conjugate through the endosomal/lysosomal pathway requires an appropriate release mechanism for the drug. Considering the potential of a large battery of lysosomally active proteases, Kopecek and co-workers studied the prerequisites of incorporating peptidyl spacers that are cleaved by cathepsin B.[237] The tetrapeptide Gly-Phe-Leu-Gly proved to be most effective with respect to both stability in blood plasma and rate of hydrolysis in the presence of cathepsins. Preclinical in vivo studies with doxorubicin–HPMA conjugates were performed in various animal models, that is, B16F10 melanoma,[238] M5076,[236] LS174T human colorectal xenografts,[236] and sensitive and resistant human ovarian carcinoma models[239–243] in which polymer-bound doxorubicin showed enhanced efficacy relative to the parent drug. It became apparent that the in vivo activity of this conjugate is correlated with the levels of cathepsin B found in tumor cells and tumor tissue.[169] Due to the promising results obtained in in vivo animal models, PK1 became the first HPMA-based macromolecular prodrug to be clinically assessed (Section 4). In the following years, several approaches were undertaken to improve the PK1 system by: 1) modifying the molecular mass and/or

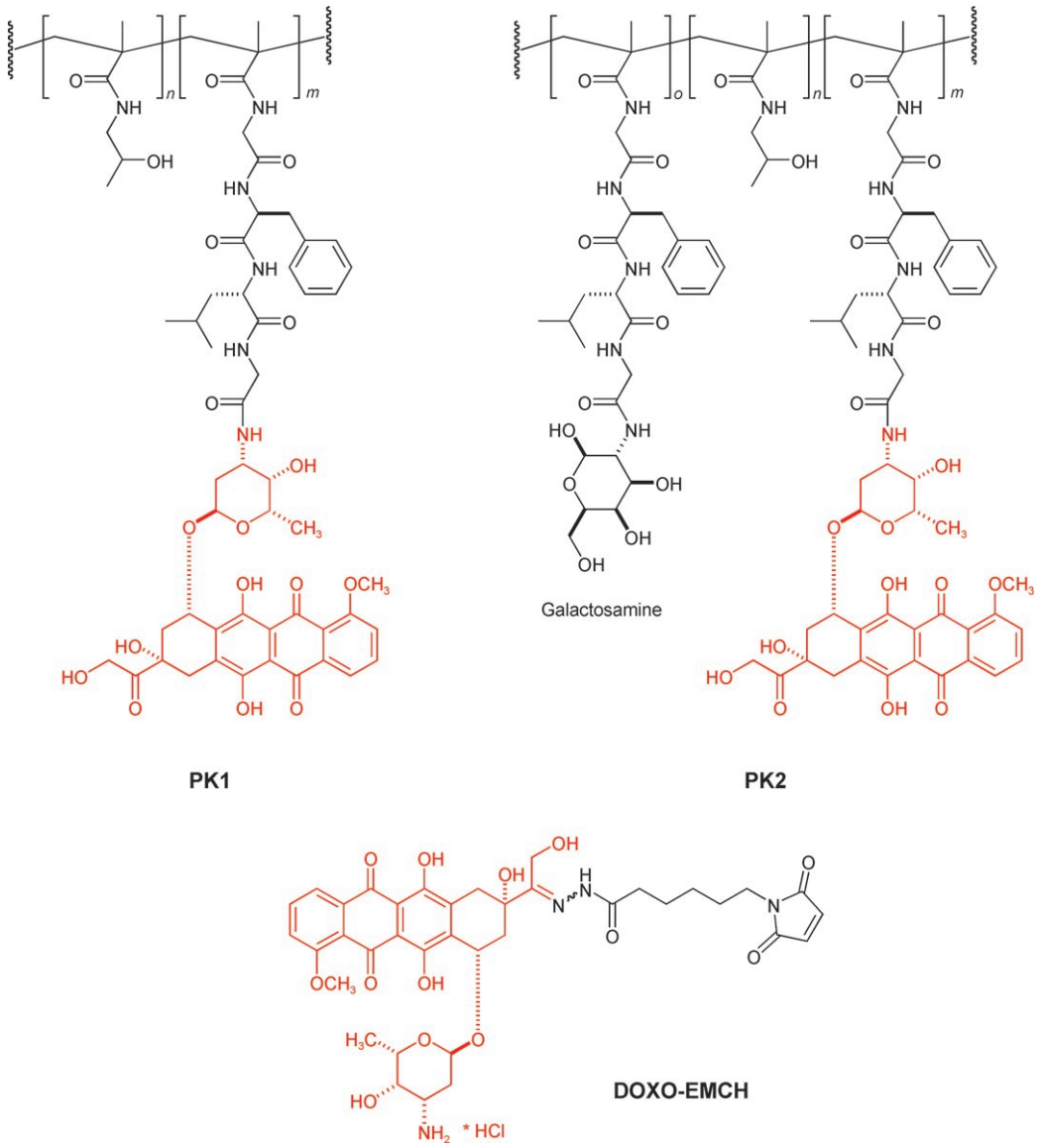


Figure 21. Structures of the clinically assessed doxorubicin prodrugs PK1, PK2, and DOXO–EMCH (INNO-206), an albumin-binding prodrug (the drug is highlighted in red).

topology of the polymer, 2) replacing the cathepsin-cleavable peptide spacer with an acid-cleavable hydrazone bond, or 3) employing PK1 in a PDEPT strategy as described below.

Because HPMA copolymers are non-biodegradable, the relatively low molecular weight of 28 kDa was chosen for PK1 to ensure slow renal clearance, thus preventing side effects that might result from unwanted long-term tissue accumulation of the polymeric carrier. As a negative result, PK1 does not display optimal tumor targeting: Cross-linked high-molecular-weight homologues of PK1 with molecular weights ranging from 22–1230 kDa were synthesized by Kopecek et al. with the aim of enhancing the circulatory retention.[244,245] To circumvent accumulation in various organs of the body, enzymatically cleavable cross-links were incorporated in the architecture of the polymer which render the polymer biodegradable. After evaluating the body distribution of the cross-linked HPMA copolymer–doxorubicin conjugates, Kopecek et al. found that the half-life of a 1230-kDa conjugate in the blood was 5-fold higher than for a 22-kDa conjugate. In addition, a concomitant decrease in the elimination rate from the tumor by a factor of 25 was observed.[245] In vivo studies performed in mice bearing human ovarian OVCAR-3 xenografts revealed that the high-molecular-weight prodrugs were significantly more efficacious than their congeners of lower molecular weight (Table 9). Unfortunately, these conjugates have not been tested in a direct comparison with free doxorubicin or with PK1, but one can assume that the antitumor efficacy of PK1 (28 kDa) would be comparable to the 22 kDa conjugate. Other attempts to improve the system by altering the topology of the carrier polymer were less successful.[246]

Table 9. Cross-linked HPMA copolymer conjugates of doxorubicin: impact of molecular weight on in vivo efficacy.[245]

Conjugate M_w [kDa]	M_w/M_n	DOX [mol%]	DOX [wt%]	Tumor volume[a]
22	1.3	1.38	5.25	1100
160	3.6	1.34	5.10	480
895	3.3	1.14	4.34	260
1230	5.2	1.01	3.84	250
Control	–	–	–	2600

[a] OVCAR-3, day 34.

Non-targeted polymeric doxorubicin in which the drug is bound through either acid-cleavable carboxylic hydrazone bonds or *cis*-aconityl spacers were reported by the research groups of Kopecek and Ríhová, et al.[247–253] Incubation studies at different pH values revealed that all conjugates were relatively stable in buffer solution at pH 7.4.[248,250] At pH 5.0, the prodrugs with incorporated hydrazone bonds released doxorubicin with half-lives of approximately 5 h, whereas the doxorubicin bound through *cis*-aconityl spacers was released at a much slower rate (half-lives $>$ 50 h).[250] In an EL4 T-cell lymphoma xenograft model, the acid-labile polymer conjugate (hydrazone linker) proved to be significantly more active than free doxorubicin as well as PK1.[248] Furthermore, in vitro studies against various cell lines[249] revealed that the cytotoxic effect of the acid-cleavable prodrugs is up to two orders of magnitude higher than PK1, and in some cell lines, even comparable to the free drug.

Another attempt to improve the therapeutic potency of PK1 was realized in a PDEPT approach. Satchi et al. developed HPMA copolymer-bound cathepsin B that was co-administered with PK1.[126] The PDEPT combination proved to be more efficacious than PK1 alone in a B16F10 melanoma model and showed antitumor activity against a COR-L23 xenograft, whereas PK1 was not active. The examples mentioned above show that the development of non-targeted HMPA-based prodrugs of doxorubicin did not come to an end with PK1 entering clinical trials.

Prothecan, a 40-kDa PEG-modified version of camptothecin, was the first drug conjugate with poly(ethylene glycol) that has been assessed clinically (Figure 22 and Section 4). PEG is one of the most versatile polymers for medical applications, characterized by its outstanding chemical properties including chemical inertness of the polyether backbone and its excellent solubility in aqueous media. Furthermore, PEGs are nontoxic, non-immunogenic, and non-biodegradable, making them suitable for the modification of various biologically active compounds.

Conjugating the 20-OH position of camptothecin through a glycine spacer with PEG having a molecular weight of 40 kDa[254–256] rendered excellent water solubility relative to free camptothecin, which is practically water insoluble. In addition,

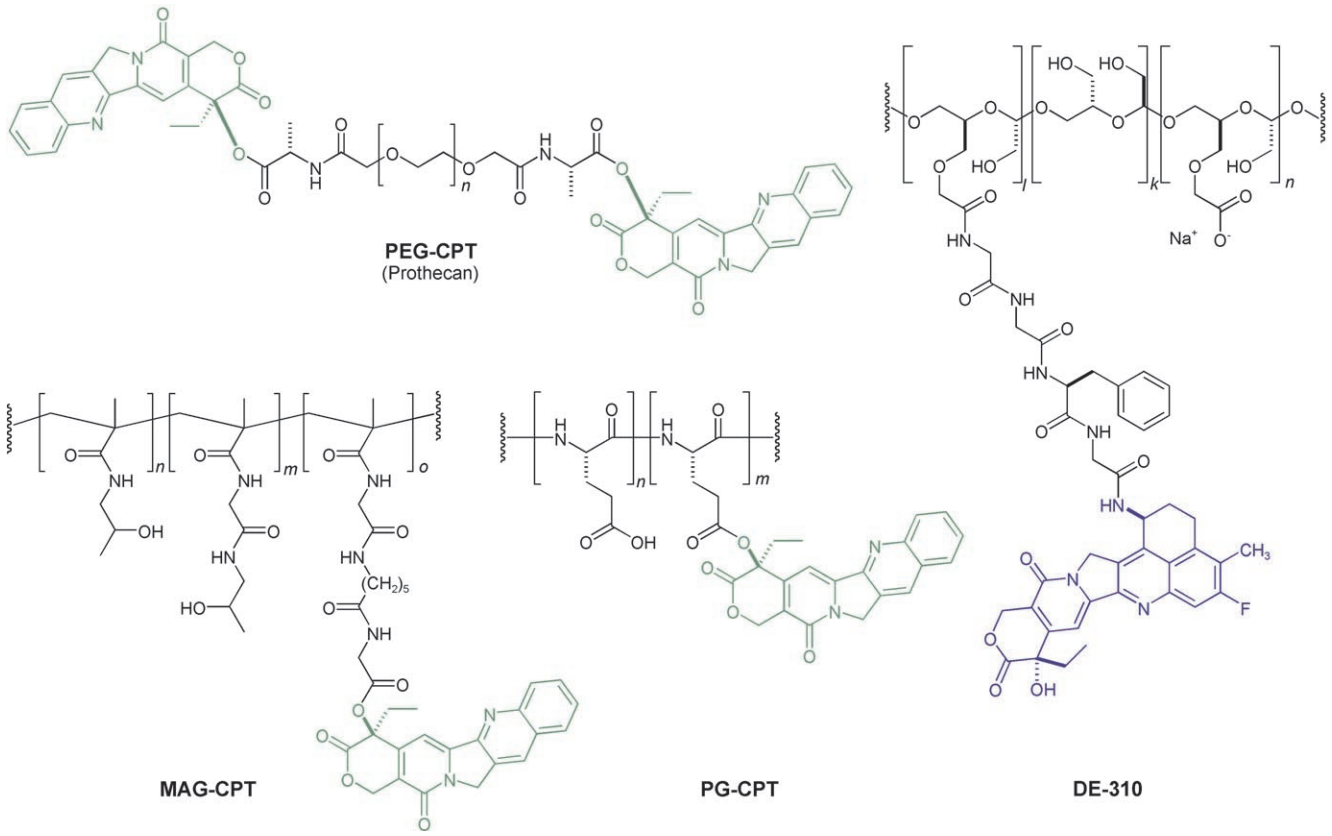


Figure 22. Structures of the clinically assessed CPT prodrugs PEG–CPT, MAG–CPT, and PG–CPT (with the drug highlighted in green) and DE-310, a carboxymethyldextran conjugate with DX-8951f, a CPT derivative (highlighted in blue).

esterification of the 20-hydroxy group of camptothecin stabilizes the drug in its active lactone form (closed E-ring), which otherwise tends to hydrolyze under physiological conditions and leads to the inactive hydroxycarboxylic acid form. Release of camptothecin very likely occurs through liberation of the camptothecin–glycinate ester in a first step, followed, not as expected by direct hydrolysis to camptothecin, but instead by formation of a six-membered morpholine-2,5-dione ring that is obtained through an intramolecular reaction of the amino group of the glycine moiety with the lactone ring of camptothecin. This species then hydrolyzes to camptothecin and camptothecin carboxylate.[257]

Clinical development was not pursued with this prodrug but with an analogous PEG–camptothecin conjugate (Prothecan) in which the glycine spacer was substituted by an alanine spacer which showed improved stability in human blood plasma. Preclinical results with Prothecan showed enhanced antitumor efficacy in animal models of human cancers in comparison with currently marketed products.[255]

Xyotax (CT-2103), a poly(L-glutamic acid) conjugate of paclitaxel (Figure 23), is probably the most successful drug–polymer conjugate to date and is meanwhile undergoing phase III trials (Section 4). Compared with the two drug–polymer conjugates mentioned above, Xyotax has a high loading ratio (~37 wt% paclitaxel) with paclitaxel being linked through its 2′-OH group to the poly(glutamic acid) backbone. Furthermore, in contrast to HPMA or PEG, the poly(glutamic acid) backbone of Xyotax is biodegradable; in vitro and in vivo studies have shown that paclitaxel and paclitaxel glutamic acid derivatives are released which, in part, appears to be due to cleavage by cathepsin B.[258]

In recent years, several attempts were made with dendrimers or dendritic polymers as high loading capacity carriers for anticancer drugs,[259] but not all of them proved to be beneficial. Problems associated with the use of perfect (monodisperse) dendrimers are clearly related to the synthetic difficulties of achieving sufficiently high molecular masses for passive tumor targeting. Furthermore, attaching drugs at the periphery of the dendrimer can lead to unpredictable aggregation.[260] Recent research efforts to combine the advantages of linear poly(ethylene glycol) and dendritic structures resulted in the development of interesting hybrid materials of various architectures such as dendronized linear polymers,[261] starlike PEG with terminal dendrons,[262] and so-called "bow-tie" hybrids.[263,264] The bow-tie dendrimers synthesized by Gillies et al. consist of two covalently attached polyester dendrons, each bearing different terminal groups. One dendron is usually grafted with solubilizing PEG, the other can be loaded with drugs. By using PEG chains of various lengths, it was possible to synthesize well-defined PEG–dendrimer hybrids with low polydispersity and different molecular weights.[263] Owing to their lack of toxicity and an advantageous biodistribution profile (urinary excretion and significant accumulation in tumor tissue), some of these polymers were considered as suitable carriers for anticancer drugs.[123] Recently, the synthesis of a bow-tie dendrimer conjugated with doxorubicin was reported.[265] The prodrug is based on a PEGylated dendritic scaffold (45 kDa) and contains eight PEG chains (5 kDa) and up to 16 molecules of the drug (8–10% *w*/*w*), the latter attached to the dendritic core by acid-sensitive carboxylic hydrazone bonds (Figure 24). In an in vivo experiment with C-26 tumor-bearing mice, a single i.v. application of the prodrug (20 mg kg^{-1} doxorubicin equivalents) produced complete tumor regression with a 60-day survival of 100%, whereas no cures were observed with free doxorubicin close to its maximum tolerated dose (MTD) of 6 mg kg^{-1}. Further preclinical assessment is needed

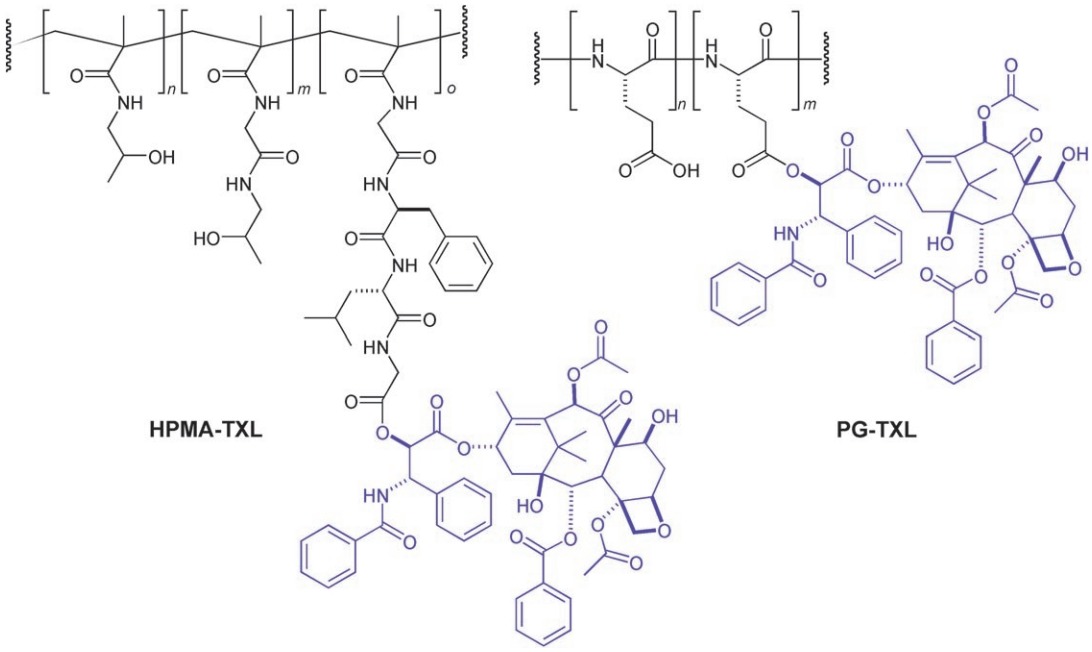


Figure 23. Structures of the clinically assessed paclitaxel prodrugs HPMA–TXL and PG–TXL (Xyotax) (the drug is highlighted in blue).

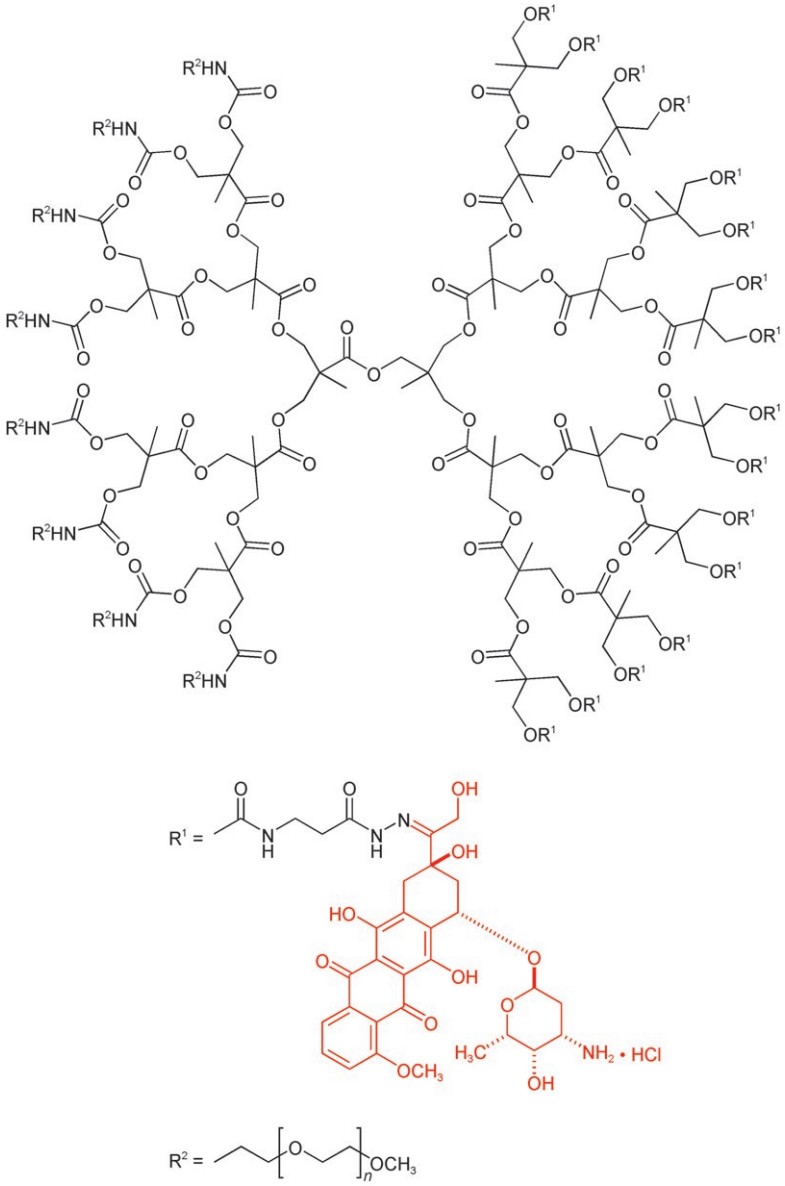


Figure 24. Structure of a PEGylated bow-tie dendrimer loaded with doxorubicin (highlighted in red) through an acid-sensitive hydrazone bond.

and will help to evaluate the antitumor potential of the most successful dendrimer-based prodrug so far.

Receptor targeting

An interesting candidate for receptor targeting is the low-molecular-weight prodrug EC145 (Figure 25) developed by Leamon et al.[266,267] that is composed of the Vinca alkaloid desacetylvinblastine monohydrazide (DAVLBH, a depolymerization inhibitor) linked to folic acid by a reducible disulfide bridge. The water solubility of the folate conjugate was improved by introducing solubility-promoting polar arginine and aspartic acid into the spacer, while the disulfide bond is necessary for the reduction-mediated drug release from the folate conjugate inside the endosomes.[268] The conjugate EC145 was found to be more active and better tolerated in in vivo preclinical studies than its acid-sensitive precursor EC140 (the analogous acetyl hydrazone derivative),[269,270] and was furthermore better tolerated than the active drug DAVLBH itself. The MTD of the conjugate EC145 was at least 2-fold higher than for DAVLBH [1 μmol kg^{-1} (0.8 mg kg^{-1})]. In addition, a significantly superior antitumor efficacy was observed. Therefore, EC145 was evaluated in a phase I study (Section 4).[271]

Following Ringsdorf's vision, receptor-targeting strategies have also focused on carriers in which a suitable ligand is bound to a polymer. Two doxorubicin conjugates were designed to selectively enter hepatocytes by binding to the asialoglycoprotein receptor (ASGPR) with subsequent internalization and degradation of the carrier in endosomes and/or lysosomes. In a study on the needle biopsies of 60 human hepatocellular carcinomas, the ASGPR was histochemically detected in 80% well-differentiated and in 20% poorly differentiated forms of the tumor[72] which forms the basis for exploiting this receptor as a molecular target for the selective delivery of drugs to hepatocellular carcinoma.

In line with this rationale, doxorubicin was coupled to the enzymatically cleavable tetrapeptide Gly-Phe-Leu-Gly linked to *N*-(2-hydroxypropyl)methacrylamide that contained additional 2.0 wt% galactosamine bound to the polymer backbone (Figure 21).[71] Preclinical studies have shown that this conjugate, referred to as PK2, delivers doxorubicin preferentially to the liver.[272–274] In an-

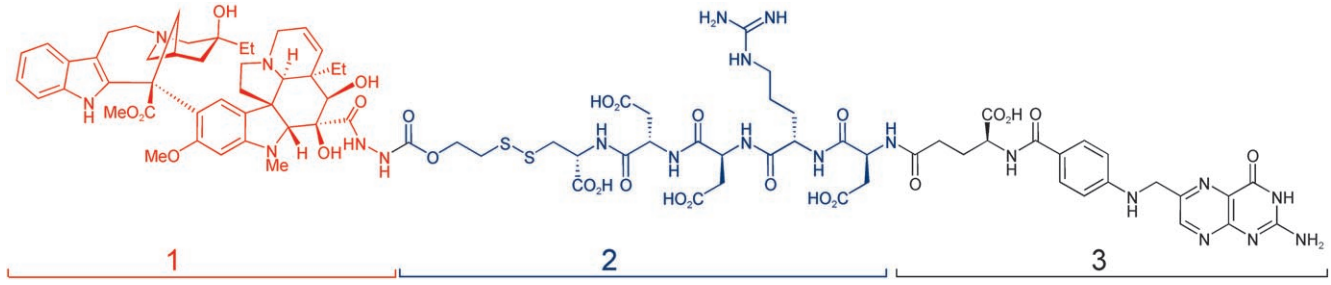


Figure 25. Structure of the folate–vinblastine prodrug EC145, containing desacetylvinblastine monohydrazide DAVLBH (1), the disulfide linker (2), and folic acid (3).

other approach, the (6-maleimidocaproyl)hydrazone derivative of doxorubicin was coupled to a thiolated form of lactosaminated human albumin (L-HSA) (Figure 26).[70,82,275] The resulting conjugate L-HSA–DOXO achieved very efficient targeting of the drug to the livers of treated mice, with doxorubicin concentrations reaching levels 7–20-fold higher than those raised in extrahepatic tissues.[275] In further experiments against hepatocellular carcinoma induced in rats by *N*,*N*-diethylnitrosamine, L-HSA–DOXO, at a dose of $4\times1\ mg\,kg^{-1}$ significantly inhibited tumor growth without decreasing body weight (Figure 27). In contrast, free doxorubicin administered at the same dose as the coupled drug did not affect tumor growth, and produced a significant decrease in the body weight of the treated animals.[70] Experiments in healthy rats have shown that even a dose of $4\times2\ mg\,kg^{-1}$ L-HSA–DOXO, twice that used in the therapeutic model, produces essentially no liver toxicity, indicating an excellent therapeutic index for the novel conjugate,[82] which is currently being developed for evaluation in a phase I trial. PK2 has been investigated in a phase I clinical trial, with two partial remissions and one minor response noted but it is not undergoing further clinical assessment.[71]

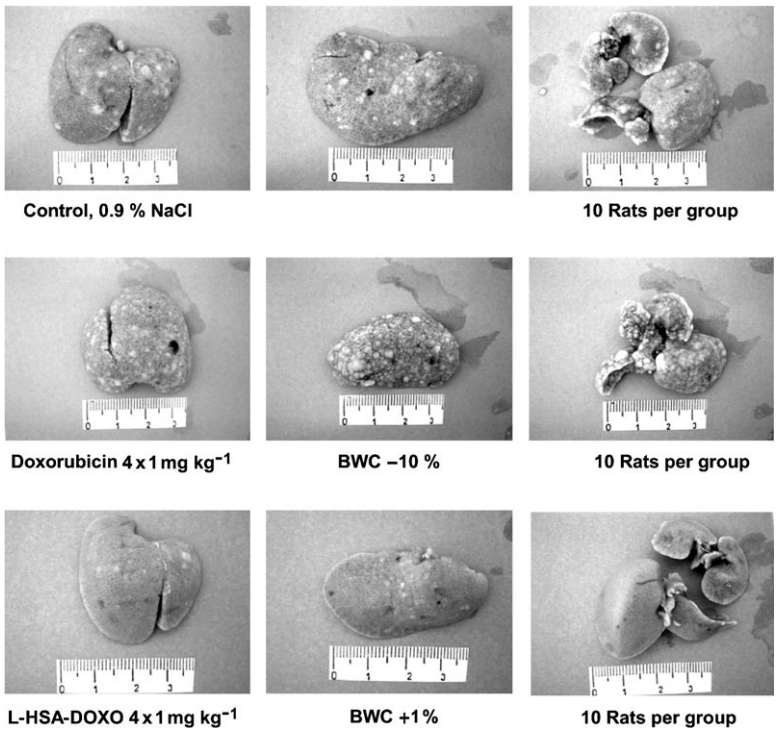


Figure 27. Efficacy of doxorubicin and an acid-sensitive doxorubicin conjugate with lactosaminated albumin (L-HSA–DOXO) in a chemically induced hepatocellular carcinoma model. Representative images of liver tumors for the control-, doxorubicin-, and L-HSA–DOXO-treated groups.

Comparison of active and passive targeting strategies

There are only a few examples available that allow a comparison of the potential of an active or passive targeting strategy using an identical prodrug system. An instructive example is the fate of the (6-maleimidocaproyl)hydrazone prodrug of doxorubicin (DOXO–EMCH) bound either to the mAb BR96 or to endogenous albumin (Figure 28). In the BR96–doxorubicin conjugate, eight molecules of DOXO–EMCH are bound to the chimeric mAb BR96 that is specific for Lewis Y, an antigen abundantly expressed on the surface of several human carcinomas, especially breast cancer.[276] Alternatively, in a passive targeting approach, DOXO–EMCH is bound in situ to cysteine 34 of circulating albumin after intravenous application.[115,277]

Both prodrugs have been investigated preclinically and clinically. A shift in the MTD for both prodrugs over free doxorubicin was noted in preclinical mice models: a ~4.5-fold increase for DOXO–EMCH (i.v.)[115] and a 2.5-fold increase for the BR96–doxorubicin conjugate (i.p.).[115,276] In addition, both formulations were superior to doxorubicin and were able to induce complete remissions in the tumor models studied.[115,276] The doses needed to achieve complete remissions were higher for DOXO–EMCH than for the BR96–doxorubicin immunoconjugate.

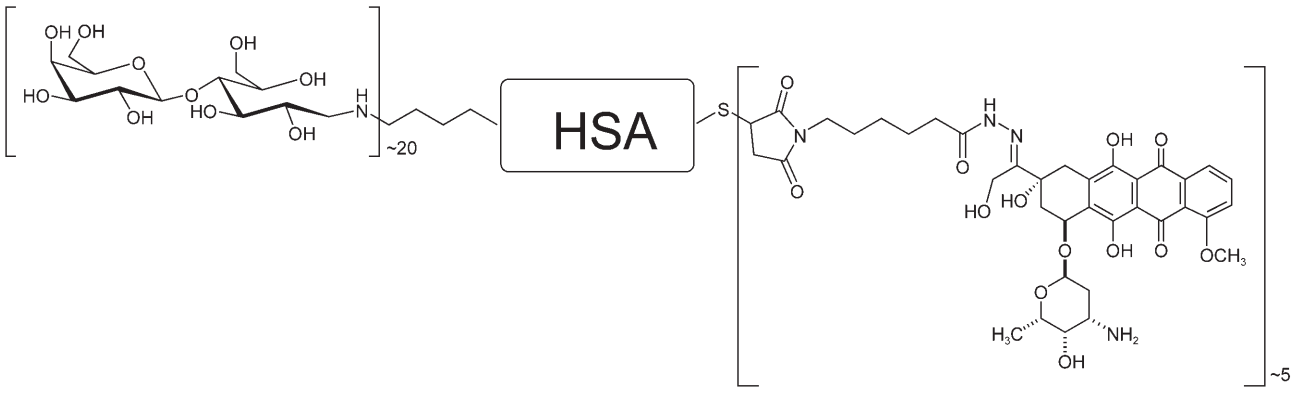


Figure 26. Structure of L-HSA–DOXO, a lactosaminated albumin conjugate bearing an average of 20 molecules of galactose and 5 molecules of DOXO–EMCH that targets the asialoglycoprotein receptor.

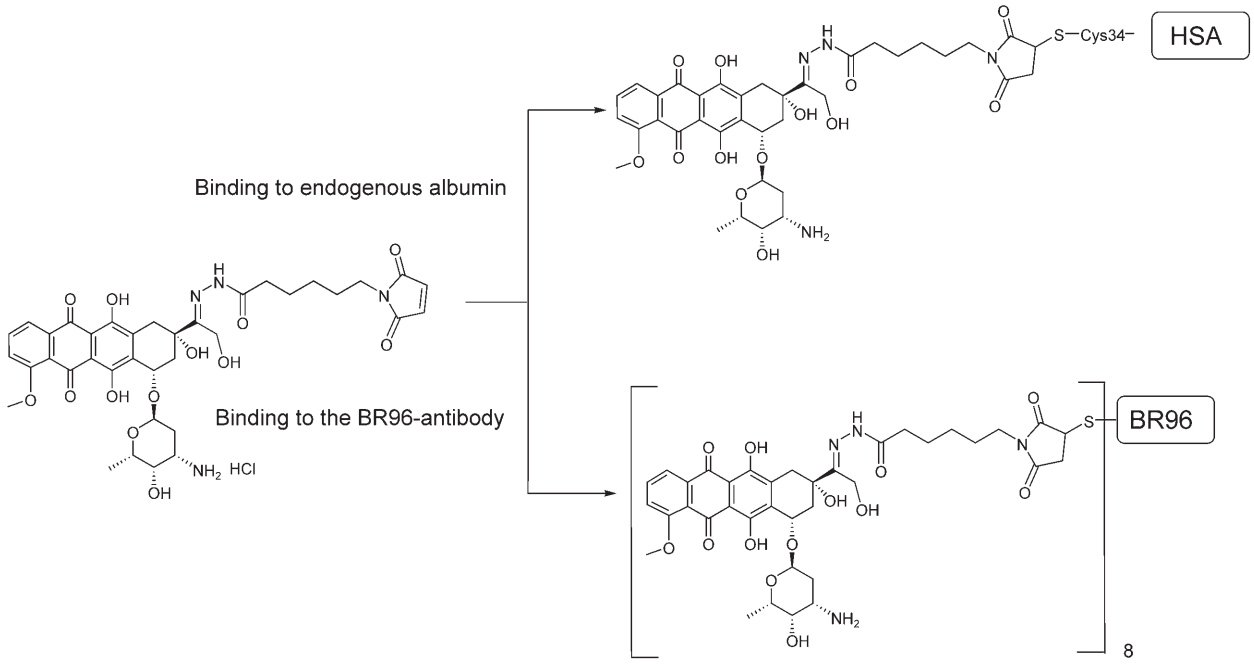


Figure 28. The (6-maleimidocaproyl)hydrazone derivative of doxorubicin (DOXO–EMCH), bound either to the mAb BR96 or to endogenous albumin, has been evaluated in clinical trials.

In clinical trials the shift in the MTD for DOXO–EMCH correlated with that observed preclinically (260 mg m^{-2} doxorubicin equivalents for DOXO–EMCH could be administered as the MTD to humans relative to a standard dose of doxorubicin of 60 mg m^{-2}), but not for the BR96–doxorubicin immunoconjugate, for which the MTD was already reached at 15 mg m^{-2} doxorubicin equivalents. This was due to severe gastrointestinal toxicity, which was probably caused by cross-reactivity with the respective normal tissue expressing the target antigen. Only limited antitumor activity has been observed in antigen-positive breast cancer patients.[278] DOXO–EMCH showed antitumor efficacy in a phase I trial and is in phase II trials for the treatment of small-cell lung cancer (Section 4 for details).

4. Prodrugs of Clinical Relevance

Several carrier-linked prodrugs with anticancer drugs have been or are being evaluated in clinical trials, and their structures are shown in Figures 21, 22, 23, 25, 29, and 30. We recently reviewed the clinical studies with these prodrugs in detail.[279] In this section we therefore restrict our discussion to a brief and comparative analysis of the clinical data. Tables 10–13 highlight their stage of development, the dose schedules, the dose-limiting toxicities (DLTs), and the number of responses in the clinical phase studies.

To date, Mylotarg, a conjugate of the cytotoxic antibiotic calicheamicin and an anti-CD33 humanized antibody, is the only macromolecular prodrug that has received market approval. Since 2003 it has been used as a single agent or in combination with current chemotherapy for the primary treatment of acute myeloid leukemia (AML). The linkage between the drug and the antibody contains two labile bonds: a carboxylic hydrazone and a sterically hindered disulfide (Figure 16). Following binding of Mylotarg to the CD33 antigen of AML cells and endocytosis, the acid-sensitive hydrazone bond is presumably responsible for the intracellular cleavage site of calicheamicin, which diffuses into the nucleus and induces DNA damage.[280]

Despite encouraging preclinical results with several immunoconjugates that use clinically established anticancer agents as drugs, the clinical antitumor efficacy of these drug–antibody conjugates has been modest.[13] There is a clear trend to use highly cytotoxic agents such as calicheamicin (the drug used for Mylotarg), maytansinoids, or auristatins in the development of modern drug–antibody conjugates. Five drug–antibody conjugates with such active agents are currently being evaluated in phase I or II studies: three antibody conjugates with the maytansinoid derivative DM1 (huN901–DM1, a DM1 conjugate with an anti-CD56 antibody for the treatment of small-cell lung cancer, MLN-2704, a DM1 conjugate with an anti-prostate-specific membrane antigen (PSMA) antibody for the treatment of prostate cancer, and huC242–DM4, a drug–anti-CanAg antibody conjugate with DM1, which is an optimized conjugate of C242–DM1 that has been evaluated in phase I studies); the calicheamicin conjugate CMC-544 that binds to CD22 on B-cell lymphomas; and finally SGN-35, an auristatin conjugate with an anti-CD30 antibody for the treatment of Hodgkin's disease and other CD30-positive hematologic malignancies (Figure 29).

The drug–antibody conjugates mentioned above were prepared with humanized or chimeric antibodies which, in most cases, avoid the problem of immune reactions (human anti-mouse antibody (HAMA) immune response). It is too early to estimate the clinical impact of these conjugates, but a number of objective responses have been achieved in phase I clinical trials (Table 11).

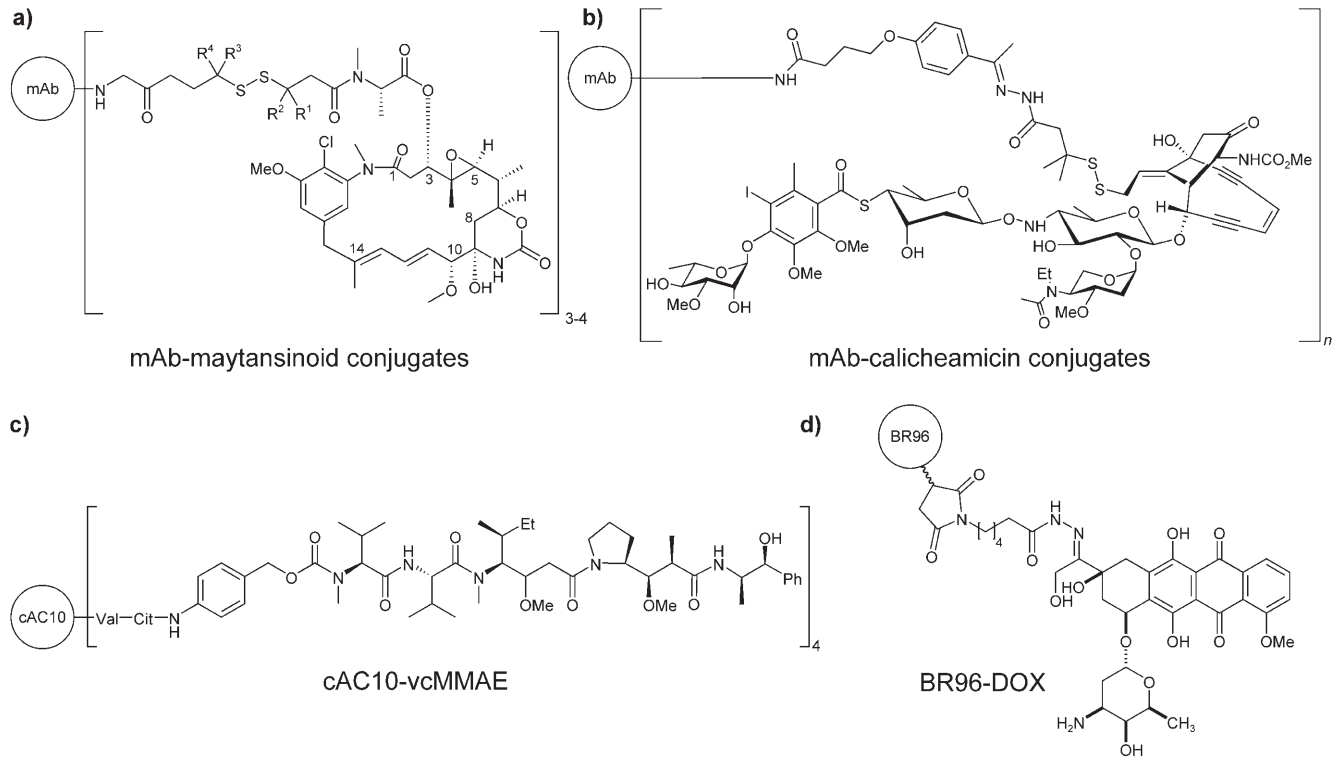


Figure 29. Drug–antibody conjugates in clinical trials. a) mAb–maytansinoid conjugates: huC242–DM1: mAb = huC242, $R^1 = R^2 = R^3 = H$, $R^4 = Me$; huC242–DM4: mAb = huC242, $R^1 = R^2 = Me$, $R^3 = R^4 = H$; huN901–DM1: mAb = huN901, $R^1 = R^2 = R^3 = H$, $R^4 = Me$; MLN2704–DM1: mAb = MLN2704, $R^1 = R^2 = R^3 = H$, $R^4 = Me$. b) mAb–calicheamicin conjugates: CMC–544: mAb = G5/44, $n = 5–7$; Mylotarg: mAb = P67.6, $n = 2–3$. c) mAb–monomethyl auristatin E conjugates: cAC10–vcMMAE: mAb = cAC10. d) mAb–doxorubicin conjugates: BR96–DOX: mAb = BR96.

Table 10. Drug–antibody conjugates in clinical trials.

Immunoconjugate	Indication	Target	Originator/Development stage	Licensee/Development stage
huN901–DM1, a humanized mAb conjugated with the cytotoxic agent maytansinoid DM1	NSCLC[a]	CD56	ImmunoGen (USA)/Phase I/II	Vernalis (UK)/Phase I/II
MLN-2704, a humanized mAb conjugated with the cytotoxic agent maytansinoid DM1	Prostate cancer	PSMA[b]	Millennium Pharmaceuticals (USA)/Phase I/II	BZL Biologics (USA)/ Phase II ImmunoGen (USA)/ Phase II
C242–DM1, a humanized mAb conjugated with the cytotoxic agent DM1	Colorectal and pancreatic cancer NSCLC	CanAg	ImmunoGen (USA)/ Discontinued	–
Mylotarg (gemtuzumab ozogamicin), a humanized mAb conjugated with calicheamicin	Acute myelogenous leukemia (AML)	CD33	Wyeth (USA)/Launched	UCB (Belgium)/Launched
BR96–DOX, a chimeric mAb chemically linked to doxorubicin	NSCLC Breast cancer	Lewis Y	Bristol-Myers Squibb (USA)/ Discontinued	Seattle Genetics (USA)/ Discontinued

[a] Non-small-cell lung cancer. [b] Prostate-specific membrane antigen.

Besides drug conjugates with antibodies, several drug–polymer conjugates that follow a passive targeting strategy have been or are being evaluated in clinical trials. In contrast to drug–antibody conjugates, these drug–polymer conjugates have been realized with clinically established anticancer drugs such as doxorubicin, methotrexate, camptothecin, paclitaxel, and platinum analogues (see Tables 12, 13 and Figures 21–23 and 30 for structures). The majority of drug–polymer conjugates have been realized with *N*-(2-hydroxypropyl)methacrylamide (HPMA) and a tetrapeptide spacer (Gly-Phe-Leu-Gly) that is cleaved by lysosomal proteases such as cathepsin B. Although these conjugates are no longer under clinical assess-

Table 11. Phase I and II trials data for drug–antibody conjugates.

Immunoconjugate	Clinical study	Number of patients	Dosage [mg m^{-2}][b]	Results[c]	DLT[d]	Ref.
MLN-2704	Phase I	11	18–343	2 PR, 2 SD	Not reached	[352]
	Phase II	6	330	4 PR	NA	[352]
C242–DM1	Phase I Part A trial	37	22–295	2 MR, 4 SD, 7 PR	Reversible elevation in serum liver enzymes	[208]
	Phase I	10	22–132	1 SD	Elevation in pancreatic lipase, hypersensitivity, nausea and vomiting, and facial flushes	[353]
	Phase I	39	40–138	1 SD, 1 PR, 1 CR	Elevation of hepatic transaminases, fatigue	[354]
Gemtuzumab ozogamicin	Phase I	40	0.25–9	2 CR, 1 PR	Fever, chills, and hypertension	[190]
	Phase II	142	9	23 CR	Anemia, severe neutropenia, thrombocytopenia	[280]
	Phase II/III	>90	9	10 CR	NA	[280]
BR96–DOX	Phase I	66	66–875	2 PR	Gastrointestinal toxicity, fever, elevation of pancreatic lipase, nausea and vomiting	[26]
	Phase II	14	700	1 PR	Gastrointestinal toxicity, elevation of pancreatic lipase, nausea and vomiting	[278]
	Phase II	15	700	5 SD	Gastrointestinal toxicity, primarily nausea, emesis	[355]
huN901–DM1	Phase I	32	5–75	4 SD, 1 PR	Fatigue, nausea, headache, and neuropathy	[356]
	Phase I	13	5–40	some SD was noticed	None observed	[356]
	Phase I	NA[a]	4–75/day	13 SD, 1 PR	No clinically significant myelosuppression or serious infusion reactions	[356]
	Phase II	14	60	3 SD, 2 PR	Headache and hyperthesia	[356]

[a] NA: not available. [b] With respect to drug–antibody conjugate. [c] CR: complete remission, PR: partial remission, MR: minor response, SD: stable disease. [d] Dose-limiting toxicity.

Table 12. Overview of carrier-linked prodrugs with synthetic polymers, serum proteins, or folic acid in clinical trials.

Prodrug	Current status	Highest stage reached	Company
Camptothecin prodrugs			
PEG–CPT (Pegamotecan, Prothecan), a 40-kDa PEG conjugate	–	Phase II	Enzon
MAG–CPT (PNU-166148), an 18-kDa HPMA copolymer conjugate	–	Phase I	Pharmacia/Pfizer
PG–CPT (CT-2106), a polyglutamate conjugate	Phase I/II	Phase II	Cell Therapeutics
DE-130, a 340-kDa dextran conjugate with the camptothecin derivative DX-8951	Phase I	Phase I	Daiichi Pharmaceuticals UK
Paclitaxel prodrugs			
PEG–TXL, a poly(ethylene glycol) conjugate	–	Phase I	Enzon
PG–TXL (Xyotax), a 52-kDa polyglutamate conjugate	Phase III	Phase III	Cell Therapeutics
HPMA–TXL (PNU-166945), an *N*-(2-hydroxypropyl)acrylamide copolymer conjugate	–	Phase I	Pharmacia
Doxorubicin prodrugs			
PK1 (FCE28068), a 30-kDa *N*-(2-*h*ydroxypropyl)acrylamide copolymer conjugate	–	Phase II	Pharmacia/Cancer Research UK
PK2 (FCE28069), a 27-kDa *N*-(2-hydroxypropyl)acrylamide copolymer conjugate with galactose ligands	–	Phase II	Pharmacia
INNO-206 (DOXO–EMCH), an albumin-binding prodrug	Phase II	Phase II	Innovive Pharmaceuticals
Platinum prodrugs			
AP5280, a 25-kDa *N*-(2-hydroxypropyl)acrylamide conjugate with a diammine platinum(II) moiety	–	Phase I/II	Access Pharmaceuticals
AP5346 (ProLindac), a 25-kDa *N*-(2-hydroxypropyl)acrylamide copolymer conjugate with a diamminecyclohexane platinum(II) moiety	Phase II	Phase II	Access Pharmaceuticals
Vinblastine prodrugs			
EC145, a low-molecular-weight prodrug of folic acid and vinblastine hydrazide	Phase II	Phase I/II	Endocyte

Table 13. Data from phase I clinical trials with camptothecin, doxorubicin, paclitaxel, platinum, and vinblastine prodrugs.

Drug	Ref.	Number of patients[a]	DLT[b]	Recommended dose [mg m^{-2}]	Tumor response[c]
Camptothecin prodrugs					
PEG–CPT	[357]	37 (36)	Neutropenia	116	1 PR 2 MR
	[358]	27	Neutropenia	54	2 MR
MAG–CPT	[359]	16 (11)	Cumulative bladder toxicity	68	1 SD
	[360]	23	Myelosuppression, neutropenic sepsis, and diarrhea	200[d]	
	[361]	9 (6)	Cumulative bladder toxicity		2 SD
PG–CPT	[362]	24	Neutropenia and thrombocytopenia	75	NA
	[363]	26 (25)	Thrombocytopenia and fatigue	25–35	3 SD
DE-310	[364]	27 (25)	Neutropenia, thrombocytopenia, and hepatotoxicity	7.5[e]	1 CR 1 PR 14 SD
Doxorubicin prodrugs					
PK1	[365]	36	Neutropenia and mucositis	280	2 PR 2 MR
PK2	[71]	31 (18)	Neutropenia	120	2 PR 1 MR
INNO-206	[366]	41 (30)	Neutropenia and mucositis	200–260	3 PR 2 MR 15 SD
Paclitaxel prodrugs					
HPMA–TXL	[367]	12	–	–	1 PR 2 SD
PEG–TXL	[368]	13	Neutropenia	–	NA
PG–TXL	[369]	19 (13)	Neutropenia	233	1 PR 8 SD
	[369]	11 (8)	Neuropathy	177	1 PR 1 SD
	[370]	7	Neutropenia and neuropathy	–	2 SD
	[371]	22 (19)	Neutropenia and thrombocytopenia	225	3 PR 12 SD
	[372]	21 (12[d])	Gastris, oesophagitis, neutropenia, and dehydration	70	4 CR[d] 7 CR[d]
Platinum prodrugs					
AP5280	[373]	29 (19)	Vomiting	3300[f]	5 SD
AP5346	[283]	26 (16)	Neutropenia	640[f]	2 PR 1 MR 4 SD
Vinblastine prodrugs					
EC145	[271]	22	Constipation and peripheral sensory neuropathy	–	1 PR 6 SD

[a] Values in brackets indicate those who were able to be evaluated for tumor response. [b] Dose-limiting toxicity. [c] CR: complete remission, PR: partial remission, MR: minor response, SD: stable disease. [d] Only loco-regional response was evaluated. [e] Every six weeks. [f] Pt drug equivalents.

ment, phase I and II studies have shown that no special toxicity can be attributed to HMPA, and the drug–polymer conjugates have, in most cases, shown a favorable toxicity profile. However, the anticipated broad antitumor efficacy of HPMA–drug conjugates such as PK1, the first doxorubicin–HPMA conjugate to enter clinical trails, was not observed in phase II studies. The cathepsin B-cleavable tetrapeptide Gly-Phe-Leu-Gly might not be the ideal linker considering that the antitumor efficacy of PK1 in preclinical models correlated with the expression of cathepsin B in the tumor.[169]

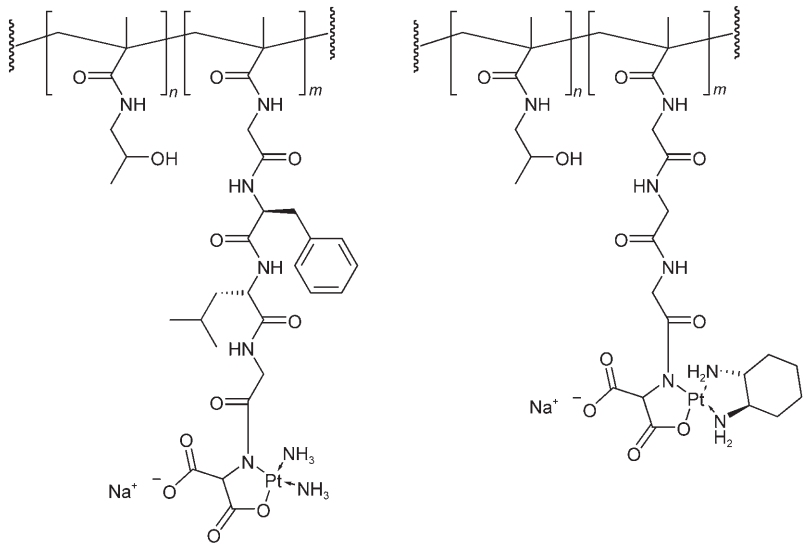


Figure 30. Structures of the clinically assessed platinum-based prodrugs AP5280 and AP5346 (ProLindac).

At present, four carrier-linked prodrugs are being assessed clinically in phase II and phase III studies: PG–TXL and PG–CPT, poly(glutamic acid) conjugates with paclitaxel and camptothecin, respectively; AP5346, an oxaliplatin conjugate with HPMA; and DOXO–EMCH, an albumin-binding prodrug of doxorubicin. The most advanced drug–polymer conjugate is PG–TXL, also known as Xyotax, in which paclitaxel is bound through its 2′-OH group to a polyglutamate carrier. Release of paclitaxel and monoglutamyl-2′-paclitaxel as well as diglutamyl-2′-paclitaxel appears to be mediated by the lysosomal protease cathepsin B.[258] Although phase I and II studies had shown a promising response rate, results from two large phase III studies (STELLAR 3 and 4) conducted in >800 patients with advanced non-small-cell lung cancer[281] have primarily shown a significant benefit in survival for women treated with PG–TXL in the STELLAR 4 trial. A phase III study (PIONEER: PG–TXL versus paclitaxel first-line monotherapy) in women with NSCLC is currently being carried out to confirm this survival advantage.[281] An analogous PG conjugate with camptothecin is currently being evaluated in a phase II study.[282]

Following successful phase I studies, a platinum conjugate with HPMA (AP5346)[283] and an albumin-binding prodrug of doxorubicin DOXO–EMCH (renamed INNO-206)[277] have entered phase II studies. Although the structures of these prodrugs are not related (Figures 21 and 30), they both depend on an acid-promoted release of the active agent. In addition, both prodrugs show a pronounced increase in the MTD relative to the free anticancer drug. AP5346 (ProLindac) is currently being evaluated in a phase II clinical study in patients with recurrent platinum-sensitive ovarian cancer, while INNO-206 is in phase II studies for the treatment of small-cell lung cancer.

Finally, one low-molecular-weight prodrug, EC145, is in clinical trials. EC145 is a conjugate of folic acid and a vinblastine derivative (Figure 25) that has recently been evaluated in a phase I trial.[271] Two regimens, a bolus (16 patients) and a 1-h intravenous infusion (6 patients) on days 1, 3, and 5 (week 1) and days 15, 17, and 19 (week 3) of a 4-week cycle were evaluated. The MTD of the bolus injection was 2.5 mg as a flat dose (~1 mg vinblastine equivalents), which is significantly less than the weekly dose of ~4–18 mg m^{-2} for vinblastine. Drug-related side effects included nausea, fatigue, constipation, and peripheral sensory neuropathy, the latter two characterizing DLTs at 4 mg EC145. Six of 22 patients showed stable disease, and one patient with refractory ovarian cancer showed a partial remission who is still on study after 36 weeks. Phase II trials in lung and ovarian cancer are planned for summer 2007 (http://www.endocyte.com).

5. Summary and Outlook

A multitude of carrier-linked prodrugs have been developed in the past 30 years with the goal of improving the therapeutic index of anticancer agents. For many of the prodrug candidates a convincing proof of concept has been obtained preclinically. Clinically, only one prodrug, a calicheamicin antibody conjugate (Mylotarg), has been approved for the treatment of a hematological cancer. Other prodrugs, primarily drug–antibody and drug–polymer conjugates, have been or are being evaluated in early clinical trials for the treatment of solid tumors. There are several reasons for the lack of clinically approved macromolecular prodrugs: a) an overestimation of the scope and relevance of preclinical data for initiating clinical trials, b) insufficient information on antigen, receptor, or enzyme expression for the tumor of the individual patient, and c) inherent problems between preclinical models and the clinical situation, for instance, large differences in the plasma stability of prodrugs between humans and the animals used in preclinical studies. For humanized drug–antibody conjugates, the cross-reactivity in most preclinical models is practically absent, an observation that does not reflect the human situation. For passive targeting strategies there is a wealth of information available on the EPR effect in preclinical models, but respective information for individual tumor types and metastases in humans is scarce.

The macromolecular prodrugs that have been evaluated clinically to date were assessed as monotherapy. It is very likely that the best clinical benefit with these prodrugs can be achieved in combination therapy with classic cancer chemotherapy or other novel approaches such as antibody-based therapy, considering that the individual prodrug addresses certain characteristics of the heterogeneous tumor.

In the future, focus on translational research and the design of rational clinical trials will be important for validating the concept of the underlying active or passive targeting strategy. Ultimately, comparative studies of carrier-linked prodrugs with current chemotherapeutic regimens will be needed for their market approval.

Keywords: antitumor agents · drug delivery · drug design · medicinal chemistry · prodrugs

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