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Summary

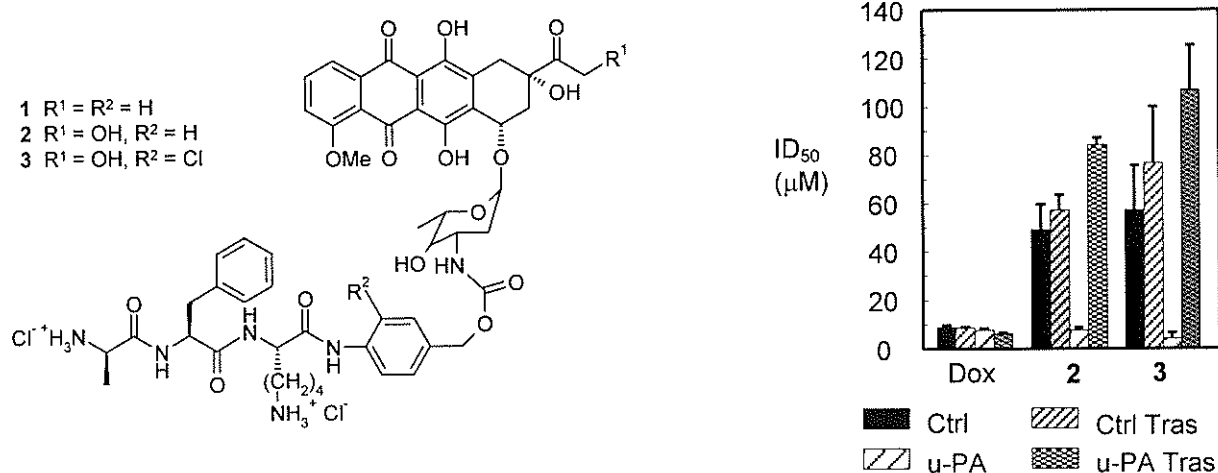
'The magic bullet': scientists have been searching for it for decades. The magic anticancer bullet should ideally kill tumor cells and at the same time spare normal cells. For years, compounds have been searched for, which fulfilled all the requirements of a successfully targeted therapy. The research described in this thesis provides chemical approaches that may contribute to improve the characteristics of prodrugs or targeted conjugates under physiological conditions.

Chapter 1 gives a general introduction on anticancer prodrug therapy. In order to improve current chemotherapeutic treatment and diminish severe side effects, several tumor-activated prodrug strategies have evolved to achieve site-specific delivery of cytotoxic anticancer agents. Antitumor prodrugs are discussed, which can be applied in the concept of prodrug monotherapy, and which are designed for direct activation or recognition by tumor-associated factors, such as hypoxia, and tumor-associated enzymes and receptors.

The first part of this thesis (up to and including chapter 5) deals with the design and synthesis of prodrugs that possess high stability against ubiquitous cleavage and that show enhanced efficiency of drug release. A successful tumor-activated prodrug must fulfill several requirements. Two essential requirements are prodrug stability against ubiquitous degradation under physiological conditions and efficient prodrug activation/recognition by the target biomolecule, which leads to efficient drug release. Prodrugs of anthracyclines (chapters 2 and 4), paclitaxel (chapters 3 and 4), and camptothecins (CPTs) (chapter 5) have served as parent anticancer drugs. For prodrug design, these parent drugs can be distinguished from one another on the basis of their functional groups that can be used as a handle for attachment to the promoity. Anthracyclines (such as doxorubicin) contain an amino function that is important for biological activity, whereas paclitaxel contains only secondary hydroxyl groups that can be used for conjugation. The camptothecins contain a tertiary hydroxyl group for conjugation.

In chapter 2, prodrugs of the anthracyclines daunorubicin and doxorubicin are described, which have been designed for activation by the tumor-associated serine protease plasmin. The prodrugs contain a self-immolative 1,6-elimination spacer (figure 1), which enables proteolytic activation by plasmin. This is in contrast to anthracycline prodrugs designed for activation by plasmin reported in the literature, which do not contain a spacer, and which are not cleaved by plasmin.

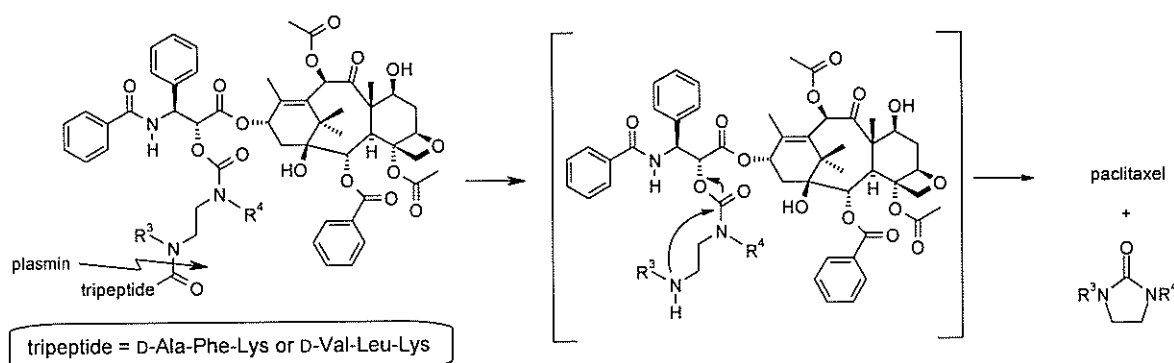
Figure 1 Spacer-containing anthracycline prodrugs and their *in vitro* selective cytotoxicity.



The prodrugs were considerably less cytotoxic than the parent anthracyclines upon incubation with seven human tumor cell lines. The prodrugs have displayed considerable *in vitro* selective cytotoxicity for plasmin-generating urokinase-type plasminogen activator (u-PA) transfected MCF-7 breast cancer cells, whereas they were considerably less toxic for MCF-7 control cells (figure 1). Both doxorubicin prodrugs showed a similar cytotoxic effect in comparison with free doxorubicin only in the u-PA transfected cells, indicating complete conversion of the prodrug by plasmin. Addition of the plasmin inhibitor aprotinin drastically increased the ID₅₀ values (drug dose that inhibits cell growth by 50% compared to untreated control cultures) in the u-PA transfected MCF-7 cells for both doxorubicin prodrugs, indicating that the observed toxicity is plasmin-mediated.

Paclitaxel-2'-carbamate prodrugs are reported in chapter 3. Up to now, reported paclitaxel prodrugs contained 2'-ester or 2'-carbonate linkages. Paclitaxel-2'-carbamates are particularly interesting because a free 2'-hydroxyl group is important for biological activity and because in general carbamate linkages are more stable *in vivo* than esters and carbonates. Several ethylene diamine spacer-containing prodrugs have been designed and prepared (scheme 1). A generally applicable route for the synthesis of paclitaxel-2'-carbamates is presented in this chapter.

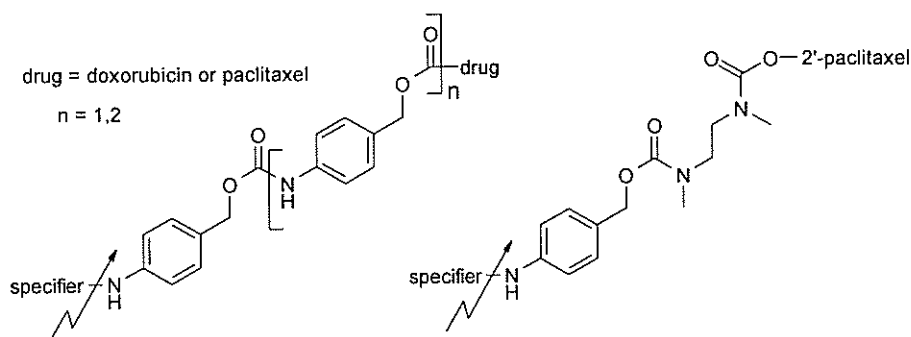
Scheme 1 Mechanism of drug release from paclitaxel-2'-carbamate prodrugs.



Biological characterization of the synthesized prodrugs revealed that if R³ is a methyl group, plasmin activation does not take place. It was also discovered that if R⁴ is a proton, the resulting 2'-carbamate is not stable. The prodrug with R³ = H and R⁴ = methyl was shown to be stable and it was capable of generating paclitaxel upon incubation with plasmin. A paclitaxel-2'-carbonate prodrug, containing a classical 1,6-elimination spacer, was also synthesized. The synthesized prodrugs displayed greatly decreased cytotoxicity in comparison with the parent drug paclitaxel.

Chapter 4 describes the design and synthesis of prodrugs of doxorubicin and paclitaxel with several novel elongated self-elimination spacer systems. A paclitaxel prodrug containing an elongated promoiety, designed for bioreductive activation under hypoxic conditions and subsequent 1,8-elimination, was shown to release paclitaxel upon chemical reduction. Model compounds with naphthalene and biphenyl-containing spacers were synthesized but self-elimination did not take place. Doxorubicin and paclitaxel prodrugs containing two or three electronic cascade 1,6-elimination spacers were synthesized (figure 2; left).

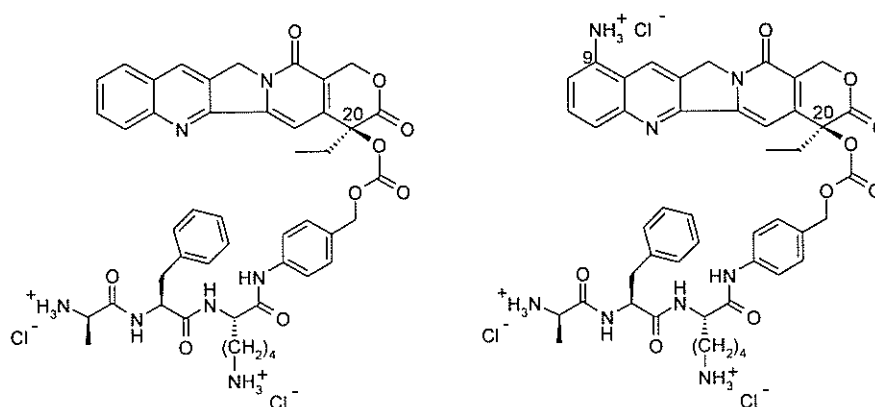
Figure 2 Prodrugs with elongated spacer systems consisting of multiple electronic cascade and/or cyclization spacers.



A novel catalytic application of N-hydroxybenzotriazole (HOBT) was found, namely for the synthesis of N-aryl carbamates through reacting a 4-nitrophenyl carbonate with an aniline derivative. Using HOBT, 1,6-elimination spacers were connected to one another via a carbamate linkage. A double spacer-containing paclitaxel prodrug was also synthesized, comprising a 1,6-elimination spacer and a bis-amine linker connected to paclitaxel via a 2'-carbamate linkage (figure 2; right). Prodrugs in which the novel spacer systems were incorporated in between a plasmin substrate and the parent drug proved to be significantly faster activated by plasmin in comparison with the corresponding prodrugs containing spacer systems of conventional length. In addition, several of these prodrugs showed a markedly decreased cytotoxicity. The elongated spacer systems reported in this chapter may be employed for contributing to enhanced drug release characteristics of prodrugs and bioconjugates.

Chapter 5 reports novel 20-carbonate prodrugs of camptothecins, designed for activation by plasmin (figure 3). Functionalization of the 20-hydroxyl group of the parent drugs should protect the prodrug against ring opening of the E-ring lactone under physiological conditions. 9-Aminocamptothecin (9-ACPT) was selectively N-protected with an allyloxycarbonyl (Aloc) group. Coupling of the promoiety to the parent drug via a carbonate linkage has been achieved by reacting the parent drug with the 4-nitrophenyl carbonate activated promoiety in the presence of 4-(dimethylamino)pyridine (DMAP).

Figure 3 Prodrugs of CPTs linked via a 20-carbonate.

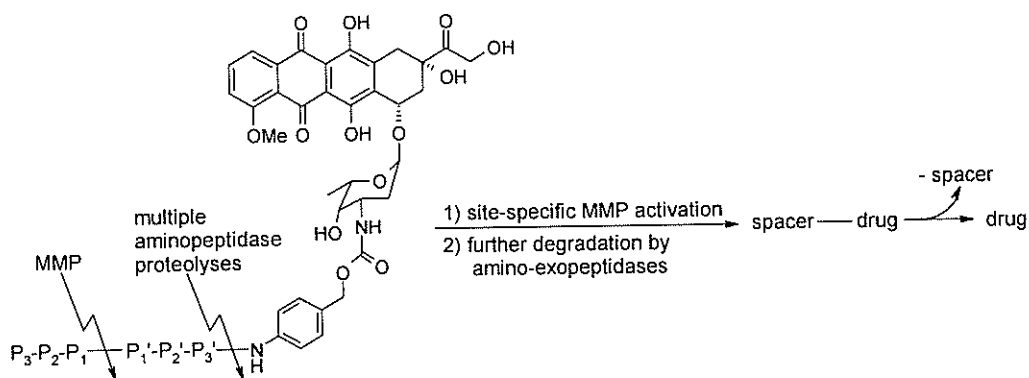


The camptothecin (CPT) prodrug was more stable against ubiquitous non-specific enzymatic cleavage than the 9-ACPT prodrug (as determined by measurement of in vitro cytotoxicity when compared to the corresponding parent drugs).

The second part of this thesis (chapters 6 and 7) describes new approaches in tumor-activated prodrug therapy that concern combinations of more than one tumor-specific moiety in a single prodrug. Such prodrug can be directed to more than one (tumor-associated) target.

Chapter 6 deals with prodrugs of doxorubicin that are designed for initial site-specific proteolytic activation by tumor-associated matrix metalloproteinases (MMPs), followed by specific or non-specific amino-exopeptidase activation (scheme 2).

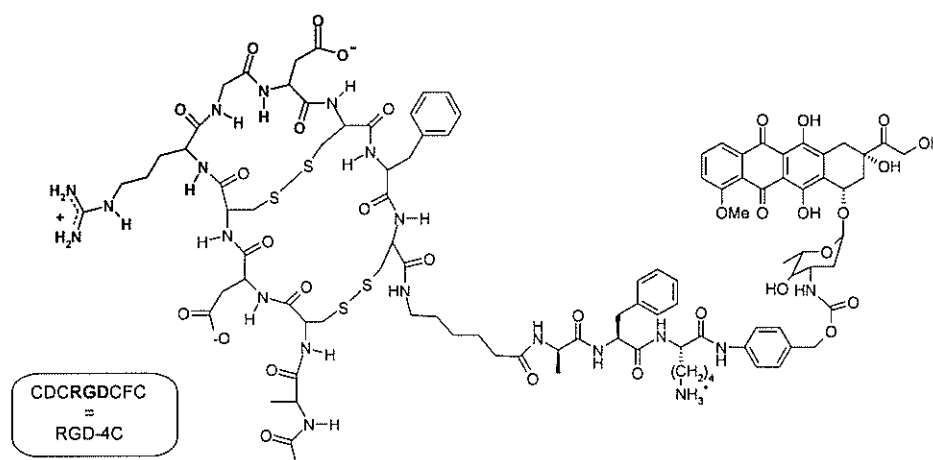
Scheme 2 Prodrugs designed for initial cleavage by tumor-associated MMPs and subsequent proteolysis by amino-exopeptidases.



A 1,6-elimination spacer was incorporated between the oligopeptide and the drug to facilitate proteolytic cleavage and make drug release more efficient. Only with the coupling agents bromotris(pyrrolidinone)phosphonium hexafluorophosphate (PyBroP) and tetramethylfluoroformamidinium hexafluorophosphate (TFFH), the anilide linkage between the oligopeptides and the spacer could be established. The prodrugs showed a greatly decreased cytotoxicity, demonstrating prodrug stability against unspecific enzymatic cleavage. Release of free doxorubicin upon specific enzymatic hydrolysis has been studied upon incubation in the presence of bacterial collagenase (known to cleave most MMP substrates) and aminopeptidases, proteinase K, or α -chymotrypsin. Under the experimental conditions, *in vitro* degradation of the peptide sequences proceeded inefficiently, resulting in low amounts of liberated doxorubicin from the prodrugs. The chosen $P_1'-P_2'-P_3'$ tripeptides may not have been proper substrates for the bacterial enzymes used in these *in vitro* experiments. Aminopeptidases present under physiological conditions *in vivo* (in mice or humans) might be able to effectively generate free doxorubicin by cleaving the final three amino acids.

Chapter 7 describes the design, synthesis, and biological evaluation of a bifunctional doxorubicin prodrug that contains a specifier with dual tumor-specificity. Both a tumor-specifically recognizable peptide (integrin binding sequence) and a tumor-selective enzymatically cleavable sequence (plasmin substrate) are incorporated in the doxorubicin prodrug (figure 4). The dual specificity motive should enhance the prodrugs' tumor-recognition potential. The bicyclic CDCRGDCFC (RGD-4C) nonapeptide selectively binds $\alpha_v\beta_3$ integrin, which is highly overexpressed on tumor endothelial cells. The D-Ala-Phe-Lys tripeptide serves for selective recognition and activation by the tumor-associated protease plasmin. To sterically separate the RGD-4C sequence from the tripeptide sequence, an aminocaproyl residue was incorporated as a spacer between the two peptide sequences, whereas a self-eliminating 4-aminobenzyl alcohol spacer was inserted between the plasmin substrate and doxorubicin (figure 4).

Figure 4 *Bifunctional integrin-targeted plasmin-sensitive doxorubicin prodrug.*



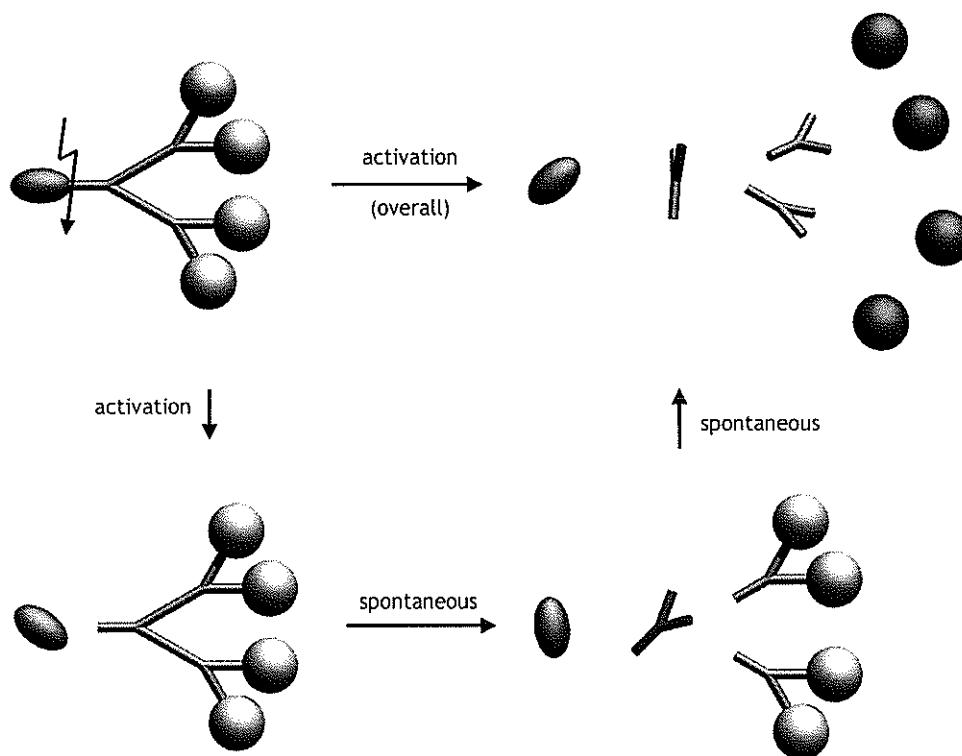
Following a convergent route, the base-labile RGD-4C fragment was coupled to an acid-labile doxorubicin fragment in the final stage to yield the desired product, which was unambiguously proven to be the desired prodrug. It was shown in two independent experiments that the RGD-4C sequence conjugated in the prodrug maintained high affinity for the $\alpha_v\beta_3$ integrin receptor. Upon incubation with plasmin, approximately one third of prodrug was converted to free doxorubicin within 60 minutes, whereas formation of free doxorubicin was completely inhibited by addition of the plasmin inhibitor aprotinin. Thus, the prodrug retained both integrin-binding and plasmin substrate properties. On the basis of the *in vitro* results described in this chapter, water-soluble derivatives of this bifunctional prodrug are considered good candidates for additional development and *in vivo* evaluation of the proposed dual targeting concept.

In chapter 8, *in vivo* results that have been obtained with two doxorubicin prodrugs (described in chapters 2 and 4) are presented. The prodrugs contain a D-Ala-Phe-Lys tripeptide specifier to be directly recognized by the tumor-associated enzyme plasmin, connected to the parent drug doxorubicin via one 1,6-elimination 4-aminobenzyl alcohol spacer (see chapter 2) or via two such spacers (see chapter 4). Both prodrugs were evaluated both *in vitro* and *in vivo* in murine EF43.fgf-4 tumor cell and human MCF-7 breast cancer cell assays, and compared to parent doxorubicin. *In vivo* toxicity (weight loss, death) and antitumor efficacy (tumor volume changes) have been evaluated. The two prodrugs showed *in vitro* toxicity towards EF43.fgf-4 cells similar to doxorubicin, indicating that prodrug conversion takes place, whereas in the MCF-7 cells, which contain low levels of plasmin, the prodrugs were less toxic than doxorubicin. Furthermore, in the presence of the selective plasmin inhibitor aprotinin, the prodrugs were substantially less toxic against EF43.fgf-4 cells, indicating a plasmin-mediated drug release (see for *in vitro* selective cytotoxicity also chapter 2), whereas in the MCF-7 cells addition of aprotinin brought about a negligible difference. In sharp contrast to doxorubicin, in both tumor models both prodrugs significantly inhibited tumor growth without discernable systemic toxicity. Doxorubicin induced considerable weight loss and death of mice, whereas mice that were given equimolar amounts of prodrug did not suffer from body weight loss or early death. In addition, both prodrugs exhibited marked anti-angiogenic activity in the EF43.fgf-4 and MCF-7 tumor models, whereas doxorubicin displayed only a slight anti-angiogenic activity or rather increased it. Remarkably, both prodrugs were effective *in vivo* against MCF-7 cells that are known to produce only low amounts of plasmin *in vitro*. It is hypothesized that plasmin is generated *in vivo* by activated endothelial cells and myofibroblasts that infiltrate the tumor. Thus, plasmin-activated prodrugs may show to be efficacious

not only against cancers that produce u-PA and plasmin, but also against cancers that do not produce these enzymes themselves. In large EF43.fgf-4 tumors, the elongated spacer-containing prodrug showed to be significantly more efficacious in reducing tumor growth than the prodrug containing a conventional spacer. This suggests that elongated spacer systems may contribute to more efficient drug release from the prodrug in vivo. The data reported herein validate in vivo the concept of tumor-targeting using plasmin-activated prodrugs, and support the clinical use of such prodrugs in new therapeutic strategies against a large series of human solid cancers.

Finally, an epilogue describes self-elimination spacer systems that release multiple leaving groups upon a single activation event. Both an electronic cascade double release spacer and a triple release spacer were developed, for which proof of principle has been delivered. Multiple generations of multiple release spacers can be coupled to one another to yield dendrimeric multiple release conjugates, which we term 'cascade dendrimers'. The multiple release spacers in these cascade dendrimers fall apart into the corresponding separate monomers and release all end groups (for example drugs) upon a single activation (for example enzymatic cleavage) (figure 5).

Figure 5 A single activation of a second generation 'cascade dendrimer' triggers a cascade of self-eliminations and induces release of all end groups. Covalently bound end groups are depicted in grey, branched self-elimination linkers in blue, and the specifier in green. Released end groups are depicted in red.



Proof of principle has been delivered for complete release of four paclitaxel molecules as end groups from a cascade dendrimer containing two generations of double release self-elimination spacers. The herein proposed 'cascade dendrimer' concept is currently incorporated in multiple release anticancer conjugates.

Outlook

It can be expected that future research will further unravel the process of tumor growth on the molecular biology level. During tumor growth, a complex communication between tumor cells and host cells takes place, and only some biomolecules that are involved in this cross talk may prove to be suitable targets for

anticancer therapy. It seems feasible to target cytotoxic drugs to these key players in tumor growth and it is likely that more tumor-associated targets will be revealed in the years to come, due to biotechnological endeavors. When considering tumor-associated proteases, novel enzymes of this class are being discovered and knowledge regarding the functional interplay between the different proteolytic systems is increasing. It will become increasingly apparent which specific enzymes are most essential for tumor growth and in highest abundance in tumor tissue. Incorporation of suitable and specific substrates for these enzymes in anticancer prodrugs may lead to multiple successful anticancer therapies. Alternatively, novel tumor-associated receptors continue to be revealed, as well as peptide sequences that selectively bind to them. These sequences may be coupled to toxic anticancer agents to target them to tumor cells expressing these receptors. Altogether, discovery of location and function of specific enzymes and receptors is expected to result in an increasing number of available handles for therapeutic intervention.

Although no enzymatically activated prodrug has been approved for clinical use yet, the enormous potential of non-toxic prodrugs in the concept of prodrug therapy is widely recognized. Prodrug monotherapy offers some advantages over two-step therapies, but substantial progress has also been made in two-step therapies such as ADEPT or GDEPT. Irrespective of whether the prodrug is used in prodrug monotherapy or in one of the two-step DEPT therapies, the prodrug must possess a number of characteristics to induce an increased therapeutic window. The prodrug should be a non-toxic derivative of the parent drug that regenerates the toxic parent drug solely at the target site. This means that the prodrug must be stable against ubiquitous hydrolysis by enzymes present in healthy tissue, organs and plasma. The components, of which a prodrug is composed, must be assembled in such a way that the product is processed exclusively in the tumor environment to release free parent drug upon arrival. Thus, it is of utmost importance to properly choose the chemistry that links the components together.

It is furthermore desirable that once the prodrug has arrived in the tumor environment, it must be efficiently activated by the target enzyme to quickly release the parent drug. Incorporation of a spacer in between targeting moiety and drug molecule can contribute to increased efficiency of prodrug activation. In numerous examples, the parent drug is not released from the prodrug if the targeting moiety is connected directly to a functional group of the drug molecule. Introduction of a self-elimination spacer may allow for fast prodrug hydrolysis by the enzyme and subsequent fast elimination to release the parent drug. Polymeric or macromolecular drug delivery is an area where self-eliminating linkers may also prove particularly useful. When parent drug molecules are attached to a polymer or dendrimer to obtain a conjugate that benefits from the enhanced permeability and retention effect (EPR), almost by definition the polymer is a large bulky moiety that may be capable of obstructing drug release from the conjugate. Properly chosen self-elimination spacer systems may potentially increase the efficiency of drug release from polymeric conjugates.

Clearly, an appropriate choice of the incorporated spacer or linker in a targeted prodrug or bioconjugate is crucial. Optimization of spacer characteristics and incorporation of self-elimination linkers in bioconjugates might improve the success of prodrugs in (tumor-activated) prodrug therapy and of targeting approaches in general. When designing receptor homing prodrugs, the mechanism of parent drug release following receptor binding should be carefully considered. Incorporation of a self-elimination spacer system in combination with a site for tumor-selective cleavage may ensure drug release. A similar approach might also be interesting for improving antibody-drug conjugates, an area in which substantial progress is currently being made.