Small Molecule Toxins Targeting Tumor Receptors

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Abstract: Targeting toxic therapeutics to tumors through receptors over expressed on the surface of cancer cells can reduce systemic toxicity and increase the effectiveness of the targeted compounds. Small molecule targeted therapeutics have a number of advantages over toxic immunoconjugates including better tumor penetration, lack of neutralizing host immune response and superior flexibility in selection of drug components with optimal specificity, potency and stability in circulation. Three major components of the targeted drug, the toxic warhead, tumor-specific ligand and the linker can influence the properties of each other and thus have to be optimized for each system. All receptor-targeted drugs are delivered inside the cells through endocytosis and undergo processing liberating the toxins in endosomes and lysosomes. Common delivery route defines a number of general requirements for each drug component. The review addresses currently known possible receptor targets and their ligands along with toxins that have been used and that have a potential to be successfully applied in tumor targeting. Linkers that are stable in circulation, but efficiently cleaved in lysosomes constitute an essential component of receptor-targeted drugs and are evaluated in greater detail.

Key Words: Peptide therapeutics, Anti-cancer drugs, Lysosomal cleavage, Drug delivery.

INTRODUCTION

Systemic toxicity of anti-cancer agents remains the main obstacle to successful therapy of cancer. There are two major reasons for the toxic side effects of drugs. The first is insufficient selectivity of the drug for the target. This problem can be corrected by better design and optimization of the drug. The second is limited specificity of the molecular target for cancer. As a result, the toxic compound hits normal cells along with the tumor. This is a very common scenario, since practically none of the currently known molecular targets is absolutely specific for cancer. To overcome this problem, we and an increasing number of other groups have examined the targeting of the toxic moieties to cancer cells through specific cell surface proteins. Thus, a targeted drug is bifunctional. One functionality targets an intracellular target while the other targets a cell surface protein receptor that allows the drug to enter only those cells that express the chosen tumor-specific receptor. This way, the drug goes through two rounds of selection and the side effects are significantly reduced. A similar principle is used in the delivery of immunotoxins that have recently shown a significant success in the therapy of some hematopoietic cancers. The major drawbacks of immunotoxins include poor tumor penetration and insufficient plasma stability that leads to premature toxin liberation in circulation and systemic toxicity. Development of neutralizing antibodies in patients receiving several rounds of therapy also reduces the potency of immunotoxins. Conjugates of small molecule ligands with small molecule toxins have the same potency potential as immunotoxins, but allow for much more flexibility in choosing the toxin with appropriate degree of toxicity and mechanism of action. They do not cause immune responses in patients and thus neutralizing antibodies are no longer a problem. They have better tumor penetration because they are small molecules and can be designed to be more stable in circulation than immunotoxins. The major difficulty in development of tumor-targeted toxins is the chemical challenge. However, significant progress has been achieved in the recent years in developing synthetic approaches and clever designs of compounds adapted for this purpose. The design of receptor-targeted drugs relies on cell biology, especially in regard to the understanding of receptor-ligand trafficking inside the cells. The fate of ligands bound to cell surface receptors has been studied extensively for a variety of receptors. The systems that were explored included various classes of membrane proteins [1], but the trafficking routes for the vast majority of cell surface receptors have turned out to be very much similar [2, 3].

Upon binding of the ligands to the cell surface receptor, the complex internalizes to endosomes and travels further to lysosomes. Many membrane proteins undergo spontaneous, ligand-independent cycling [4]. If the ligand is modified by attachment of a toxin "cargo" via an appropriate linker, they travel to lysosomes together. Here, the linker is degraded by either lysosomal proteases or by proton catalysis. The ligand for the intracellular target then needs to get out of the lysosome to reach the intended molecular target.

The design of a good small molecule targeted drug consists of three correctly chosen elements: the toxin, the ligand and the linker. In the present review, we address all the three elements summarizing the positive and negative experience that was accumulated in the field.

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TUMOR-SPECIFIC RECEPTORS AND THEIR LIGANDS

A large number of cells surface receptors have been evaluated for specific delivery of drugs into cancer cells [5]. The majority of them, however, are ubiquitous. Although they can be used to enhance selective tumor delivery to some extent, large number of those receptors is not suitable, in our view for the delivery of cytotoxic agents. We will discuss in this review only cell surface molecules that are highly over expressed in tumors and show a promise as targets that would aid in overcoming or reducing systemic toxicity.

Overexpression of receptors for neuropeptides in neuroendocrine tumors is well established [6-10]. In addition, neuropeptide receptors are frequently over expressed in other tumor types [11, 12]. Peptide hormone receptors remain very popular targets for targeted drug delivery [13], although the pattern of expression is complex, varies for different receptor subtypes and is not completely characterized for some receptors. Availability of the ligands and the straightforward chemistry of conjugate formation are just two of several reasons for that popularity. Expression of bombesin, gastrin releasing peptide (GRP), somatostatin, vasoactive intestinal peptide (VIP), neurotensin and their receptors in tumors has been recently reviewed [14]. Cancer biology of gastrin receptors is addressed elsewhere in the current volume. Receptors that show a promise as targets include bombesin (gastrin releasing peptide) receptor 2 in lung, breast, prostate, renal, ovarian, colorectal and thymic cancers, vasoactive intestinal peptide (VIP) receptor 1 in lung and breast cancers, neurotensin receptors in pancreatic and brain tumors, gastrin or CCK-2 receptor in pancreatic tumors. Somatostatin receptors are over expressed on carcinoid tumors, some breast and thyroid cancers, however, high levels of their expression in normal tissue, including peripherial nervous system and pancreas complicate their use for the delivery of toxic agents. All neuropeptide receptors are G-protein coupled (GPCR) and the trafficking pathways for GPCRs are well established [15]. They all undergo endocytosis with subsequent degradation of the ligands in lysosomes. The majority of GPCR recycle back to the cell surface, although the degree of recycling differs significantly for different receptors. Well-defined pathway makes the design of the proper targeted drug easier. Many radio labeled neuropeptides have been found useful for tumor imaging. Significant progress has been made in the field in recent years and receptor targeting with radiolabeled peptides has become a very important tool in nuclear oncology [16-19]. Diagnostic scintigraphy can be used to determine which peptide receptors are present in tumors and also to estimate the relative levels of receptor expression in cancerous and normal tissues. This information could guide the selection of the optimal peptide-toxin conjugate for the treatment of a particular tumor.

Parathyroid Hormone–Related Protein

Parathyroid hormone–related protein (PTHrP) was identified in the 1980s as a tumor product that had the ability to activate parathyroid hormone receptors and cause hypercalcemia [20]. The receptor for PTHrP found in tumors belongs to family 2 of G-protein coupled receptors. It also binds the closely related parathyroid hormone and is called PTH/PTHrP type I receptor. PTHrP peptides also bind to several other receptors and have many activities not seen with the parathyroid hormone. The whole PTHrP molecule is 139 amino acids long and the sequence is highly conserved among species. N-terminal 36 residues are highly homologous to parathyroid hormone and are responsible for binding to PTH/PTHrP receptor. Midregion peptides of PTHrP are believed to bind to other receptors and the protein itself can function as an intracrine factor and is able to enter the nucleus [21]. PTH/PTHrP receptor is expressed in most tissues and the highest levels are detected in kidney, bone and liver. Cartilaginous tumors have been found to produce significantly more PTH/PTHrP receptors than the normal tissue [22]. The level of overexpression correlated with the grade of malignancy. Very high levels of receptor expression were found in breast cancer bone metastases and it was shown to promote autocrine proliferation of breast carcinoma cells [23]. Similarly, both PTHrP and its receptor are expressed in the majority of prostate cancers and corresponding bone metastases [24]. Increased expression of both PTHrP and the receptor was reported in human glial tumors [25], although they were also detected in the neurons, reactive astrocytes and the endothelial cells of normal brain. The pattern of PTH/PTHrP receptor expression in normal tissue and tumors needs further detailed characterization. The available data suggest that it may be used for tumor targeting, but only for moderately toxic moieties because of significant expression in vital organs.

Luteinizing Hormone-Releasing Hormone

Luteinizing hormone-releasing hormone (LHRH) or gonadotropin-releasing hormone receptor has been detected in most human prostate tumor samples and in more than 50% of human breast tumor biopsies [26]. Receptor mRNA was found in both prostate cancer and benign prostatic hyperplasia [27].The protein was also detected in about 80% of human ovarian epithelial cancers [26] and in 55% of breast tumors [28, 29]. The major site of receptor expression in normal tissue is the pituitary, which may be protected from many toxic drugs by the brain blood barrier. However, this protection is not certain for all toxins and its effectiveness needs to be tested for each new compound. Receptor mRNA was also found in the kidney, thymus and spleen [30], however, the expression levels were much lower than in prostate tumors. LHRH receptor expression pattern indicates that the receptor can serve as a specific delivery vehicle for the treatment of prostate, breast and ovarian tumors. Successful inhibition of cancer cell growth and tumor growth in mice with toxins conjugated to LHRH strongly support this notion [28, 31-34].

Alpha(V)Beta(3)Integrin

Tumor vasculature has been suggested to be a promising target for anti-cancer therapy. Targeted toxins can potentially serve the purpose of cutting off the oxygen and metabolic supply to tumors by specifically eliminating endothelial cells. Peptides isolated from phage display libraries and targeting integrins are widely exploited as the delivering vehicles for gene therapy. They have also been used for targeting toxins. A conjugate of doxorubicin and tumor-homing bicyclic

peptide RGD-4C was shown to have higher efficacy than doxorubicin itself against a xenograft of human breast carcinoma in nude mice [35]. However, evaluation of the same peptide by radiolabeling with technetium-99m revealed that it has significant limitations as a delivery vehicle and as a specific marker of alpha(V)beta(3) integrin [36]. Tumor accumulation of 99mTc-RGD-4C in nude mice exhibited no statistical difference from a control peptide, possibly because of relatively low alpha(V)beta(3) integrin expression and low binding affinity of the peptide. Another cyclic peptide CNGRC selected from a phage display library and targeting tumor endothelium in xenografts was shown to be a ligand for aminopeptidase N (APN or CD13) [37]. The conjugates of the peptide with doxorubicin (Arap) and pro-apoptotic peptide KLAKLAKKLALKLA [38] were reported to have enhanced and selective *in vivo* activity against human breast cancer xenografts. However, a detailed study of the effects of DOX-CNGRC conjugate on tumor cells showed that the cytostatic effects of the drugs were CD13-independent and the conjugate was able to penetrate the cells passively [39]. The available data suggests that alpha(V)beta(3) integrin can serve as an efficient delivery target, however, higher affinity ligands and appropriate combination of toxin and linker need to be developed.

Many cell surface proteins that are over expressed by cancer cells have no known ligands. In many cases, known ligands are large proteins. The latter have been investigated as carriers for the toxins. However, their synthesis, development and application impose a number of difficulties. Random attachment of a toxin to carboxyl or amino groups of the protein can influence receptor binding. Attachment of the toxins to specific protein sites through insertion of cysteine residues with subsequent application of thiol-specific attachment can be applied in some cases, but frequently is not possible because modified protein ligands do not fold properly. In general, the chemistry of toxin coupling is restricted to reactions that can be carried out in aqueous media and mild conditions that do not cause protein denaturation. The resulting protein conjugates have limited stability in circulation and are large enough to cause an immune response, leading to production of neutralizing antibodies in patients. They also have limited penetrance into solid tumors. Development of a small molecule or peptide ligand for a particular target is the best solution that can help overcome tissue permeability, immune response and plasma stability issues. Identification of lead compounds with further optimization leading to a high affinity ligand is still a laborious, but doable task. Application of phage display libraries (T. Mori, in this volume) can be successfully applied for identification of a peptide ligand in many cases.

The following are examples of cell surface proteins that show a significant promise as receptors for specific targeting of tumor cells, but do not have small molecule ligands. We believe that the ligands can be and will be developed in the near future for the following membrane proteins and thus they may become very useful for delivering cytotoxic agents inside cancer cells.

STEAP

STEAP-1 (six transmembrane antigen of the prostate) is 339 amino acid residue integral membrane protein that is reported to be over expressed in a number of tumor types (prostate, lung, colon, bladder) [40, 41]. In normal tissue, cell surface expression of STEAP-1 is restricted to the prostate. The function of the protein has not been established and no protein or peptide ligands have been identified so far. However, *in vitro* knock-out experiments demonstrate the importance of STEAP-1 for cell proliferation. Search for proteins differentially expressed in normal and cancerous prostate cells has lead to recent discoveries of two related proteins, STEAP-2 [42] and STAMP-1 (six transmembrane protein of prostate) [43]. STEAP-2 and STAMP-1 are 51% identical to STEAP-1. Their intracellular N-termini are extended by 140 residues compared to STEAP-1. All three proteins are expressed in prostate carcinomas at much higher levels than in benign hyperplasias and thus represent exceptionally attractive cell surface diagnostic and therapeutic targets.

Mesothelin

Mesothelin (CAK1 antigen) is a glycoprotein attached to the cell membrane by a glycosylphosphatidyl inositol anchor and is over expressed in ovarian and pancreatic tumors, mesotheliomas and some but not all lung adenocarcinomas [44-46]. It is believed to be involved in cell adhesion. Conjugates of Pseudomonas exotoxin A with antibodies to mesothelin were shown to selectively inhibit the growth of mesothelin-positive tumors in mice [47].

Endoglin

Endoglin (CD105) is a major glycoprotein of vascular endothelium that plays a critical role in the binding of endothelial cells to integrins and other RGD [48]. It is a type I integral membrane protein that exists as a homodimer and forms a complex with the receptors for transforming growth factor beta. In normal human tissue, endoglin is weakly expressed on stromal cells, erythroid precursors and activated monocytes. CD105 is strongly up-regulated in the endothelium of various tumor tissues compared with that in normal tissues [49]. CD105 up-regulation was detected in a wide variety of tumors including colon, breast, brain, lung, prostate, and cervical cancer [48]. CD105 expression is suggested as a marker of high metastatic risk and poor outcome in breast carcinomas [50]. The extracellular part of endoglin is shed into the circulation and thus can neutralize parts of molecules intended for intracellular delivery. However, radiolabeled anti-endoglin monoclonal antibodies efficiently imaged primary tumors in animal models [49]. Thus, endoglin has a potential to serve as a cell surface receptor for anti-angiogenic cytotoxic tumor therapy upon development of peptide or small molecule ligands.

KCNK9

KCNK9 gene was recently found to be amplified from 3-fold to 10-fold in 10% of breast tumors [51]. The gene encodes acid-sensitive potassium channel TASK-3 (potassium channel subfamily K member 9), which was found to be over expressed from 5-fold to over 100-fold in 44% of breast tumors [51]. Normal tissue expression of TASK-3 is limited to the cerebellum with little or no mRNA detected in any other tissue [52]. The protein belongs to a large family of

two pore domain potassium channels [53, 54]. The protein chain contains four transmembrane domains and two pore-forming domains characterized by the signature motif TXGYG. The biological role of TASK-3 is largely unknown. It is over expressed at a high frequency in breast, lung, colon and metastatic prostate cancers [55]. Over expression of TASK-3 in cell lines was shown to promote tumor formation and to confer resistance to hypoxia and serum deprivation [51]. Oncogenic potential of TASK-3 was proven to depend on potassium channel function [55]. Thus, inhibitors of the channel could have therapeutic application in cancer treatment. TASK-3 molecule has a putative extracellular loop of sufficient length to raise anti-peptide antibodies that recognize the whole protein [55]. Same protein fragment could be used for identification of a smaller peptide ligand that could be used for small molecule toxin targeting.

Epidermal Growth Factor (EGF) Receptors

The epidermal growth factor (EGF) family of receptors are expressed at high levels in variety of tumors [56, 57]. Monoclonal antibodies and tyrosine kinase inhibitors specifically targeting EGFR family have advanced to clinical use and hold substantial promise of success [58-60]. The family consists of four structurally similar tyrosine kinase receptors erb1 (HER1, EGFR), erB2 (HER2, Neu, p185), erB3 (HER3) and erB4 (HER4). Receptor molecules are composed of an extracellular ligand binding domain, a single transmembrane domain and intracellular tyrosine kinase domain. Upon binding of natural ligands, EGF-like protein growth factors the receptors form homo- or hetero-dimers. Dimerization stimulates autophosphorylation in the cytoplasmic domain, which triggers intracellular signaling cascades. A mutant EGFR that lacks 267 amino acids in its extracellular domain (EGFR class III variant, or EGFRvIII) is commonly found in brain, breast, ovarian, prostate and lung cancers [57]. EGFRvIII has constitutively active tyrosine kinase, does not bind growth factor ligands and does not dimerize. Monoclonal antibodies have been developed that target different members of EGFR family and EGFRvIII. The drugs that have been developed from those antibodies can be divided into three categories: non-modified antibody molecules, fusion molecules with protein toxins and antibody conjugates with small molecule toxins. The first type has advanced as far as Phase III clinical trials, while two others are either in Phase I clinical trial or in a preclinical study. Small molecule toxins that were attached to anti-EGFR antibodies include a highly toxic maytansinoid DM1[1], adriamycin [61], doxorubicin-bound poly(L-glutamic acid) [62] and paclitaxel [63]. The conjugate with DM1 appears to be the most potent among them, however it shows some nonspecific toxicity. A peptide ligand of HER2 that mimics anti-HER2 antibody H3 lop was recently reported [64]. The peptide binds to the receptor with Kd about 200 nM and inhibits HER2 tyrosine kinase both *in vitro* and *in vivo* [65]. Identification of the peptide proves that antibodies of immunoconjugates can be substituted with smaller ligands.

[1] http://www.bioscorpio.com/database_t.htm#T

Human Mucin

Human mucin (Muc1) is highly over expressed and abnormally glycosylated (mostly underglycosylated) in carcinomas [66]. Muc1 (also called MCA, episialin, PEM, H23Ag, EMA, CA15–3) is a polymorphic, type 1 transmembrane mucin-like protein that contains a large extracellular domain incorporating a 20-amino acid repeat motif as well as a transmembrane domain and a 72-amino acid cytoplasmic tail. The function of Muc1 is not completely understood, but it has been associated with cell-cell and cell-extracellular matrix interactions, lymphocyte trafficking, and the protection of cells against microorganisms. Multiple repeats confer high immunogenicity of the mucins. As a result, many antibodies to Muc1 have been generated [67]. Antibodies to CanAg, a Muc1 variant that is strongly expressed in most pancreatic, biliary, gastric, uterine, bladder and colorectal cancers were modified by attachment of maytansinoid DM1 [68]. The resulting conjugate, Cantuzumab Mertansine developed by GlaxoSmithKline in collaboration with Immunogen is now in phase II clinical trials. Although no objective responses were observed in the phase I trial, minor responses and persistent stable disease were encouraging [69]. Cancer-specific mucins can be considered as targets for the development of specific peptide ligand-carriers due to very high overexpression in tumors. However, the development of such ligands for a glycoprotein can be complicated. An epitope that is unique for a tumor variant of a protein that consists of both glycosyl and peptide portions and thus is rather husky. Consequently, a larger ligand is needed to form a unique surface of interaction.

Cell surface proteins of hematologic malignancies have been extensively studied as targets for immunotherapy. Several immunotoxins have shown dramatic therapeutic effects in patients with hematologic neoplasms and two of them have been approved for clinical use by US Food and Drug Administration. The development of immunotoxins for hematological malignancies has been much more successful then for solid tumors, mainly because of lack of tumor penetration problem. The development of small molecule toxins for lymphomas and leukemias is less urgent then for solid tumors, since many immunotoxins show excellent promise in current clinical trials. However, the development of host neutralizing antibodies to toxins still remains a problem. Systemic toxicities are also frequently dose limiting. An excellent recent review summarizes the data on all cell surface antigens of hematopoietic cells that were tested as targets for tumor immunotherapy [70]. The ones that have been proved to be good targets include CD19, CD22, CD25, CD33 and interleukin 2 receptor [70-72]. CD2, CD3, CD5, CD7, CD30, granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR), interleukin 4 receptor, interleukin 6 receptor, urokinase receptor, interleukin 13 receptor and transferrin receptor show significant hope [70, 71].

Guanylyl Cyclase C

Guanylyl cyclase C (GC-C) is type I membrane protein that serves as a receptor for E. coli heat stable enterotoxin (ST) and two closely related peptides, guanylin and uroguanylin secreted by intestine [73]. Uroguanylin is also produced by the kidney. GC-C is expressed by primary and

metastatic colorectal adenocarcinomas, but not by any normal tissue other than intestine [74]. Radiolabeled ST peptide analog has been successfully used for imaging of human tumor xenografts in nude mice [75]. Animal studies suggested that GC-C is a good candidate for targeting colorectal tumors in humans.

The development and application of differential screens keeps unveiling novel tumor markers and targets. Although verification of each requires a significant research effort, we can expect the list of receptors potentially useful for the delivery of anti-tumor toxins to grow in the near future.

LINKERS

The choice of a proper linkage between a toxin and a carrier molecule is essential to successful drug delivery and drug release. The general properties of linkers are dictated by the drug delivery pathways. Since the vast majority of the receptor targeted drugs is delivered through endocytic pathway, the linkers are chosen based on their ability to be cleaved in endosomes and lysosomes. Several earlier reviews on targeted drug delivery via receptor-mediated endocytosis address the linkage between drug/toxin and receptor specific biomolecules [5, 26, 76-84]. The choice of proper linkers has also been discussed in related publications devoted to progress in the development of immunoconjugates [85-93], particularly cancers targeting immunoconjugates [70, 94-100], drug-polymers, and drug-fatty acid conjugates [101-104]. Comprehensive coverage of enzymatically degradable linkers can be found in several papers from "Polymers Containing Enzymatically Degradable Bonds" series [105-110].

All currently used linkers applicable for receptor-mediated delivery can be divided into three classes: direct toxin linkage, acid sensitive linkers and enzyme dependent linkers.

ACID-LABILE LINKERS

Design of acid labile linkers useful for receptor mediated drug delivery is defined by the condition that the linker should be stable in physiological pH. However it should be hydrolyzed when pH is decreased by about one pH unit, since the majority of receptor-directed drugs are delivered to endosomes (pH 6.0-6.8) or lysosomes, which have even more acidic environment (pH 4.5-5.5) [111, 112].

cis-Aconityl Linkages

cis-Aconityl spacer was first introduced as an acid sensitive linker by Shen and Ryser in 1981 [113]. Hudecz *et al.* have developed the optimum coupling condition for this linker [114] , while Blättler *et al.* described the synthesis of an improved *cis*-aconityl heterobifunctional linkage [115]. A variety of *cis*-aconityl related homo- and heterobifunctional acid sensitive cross-linking reagents, based on ortho ester, acetal and ketal functionalities, were synthesized by Srinivasachar *et al.* [116]. *cis*-Aconityl linker is used frequently and its applications have been reviewed [81, 84, 89, 101]. Hudecz *et al.* [117] addressed the issue of isomer formation during the synthesis of *cis*-aconityl linker – drug conjugate using a modified procedure originally provided by Shen and Ryser [113, 114].

The acid-sensitive *cis*-aconityl linker was applied to doxorubicin-lysozyme conjugates targeting urinary bladder [118]. The rational for this pro-drug was that low-molecular weight proteins are rapidly cleared by the kidney. Although this conjugate was not delivered through receptor-mediated pathway, the linkage could be readily adapted to receptor-targeting drugs.

The cis-aconityl linker was also used recently to connect doxorubicin to the wheat germ agglutinin (WGA), to generate a lectin-mediated drug delivery system [119]. Cis-aconityl linker was also used for non-targeted and antibody-targeted doxorubicin-polymers conjugates, based on non-degradable random copolymers of N-(2-hydroxypropyl) methacrylamide (PHPMA) [120]. Doxorubicin was connected to the cis-aconityl linker in those conjugates through the sugar amino-group and a peptide spacer was used between the polymer and cis-aconityl-doxorubicin moiety.

Esters

Because of their hydrolytic sensitivity and high risk of enzymatic cleavage in circulation, ester linkers are not used very often in the construction of pro-drugs. Recently, new taxol conjugates with arginine-based transporters were reported [121], in which the taxol molecule was connected to the octo-arginine amide derivatives through C2' or C7 hydroxyl group of taxol by forming an ester bound. Free taxol was released from conjugates by rapid hydrolysis at pH 7.4 with half-life from 1 to 214 minutes. These conjugates were designed to improve taxol solubility by producing water-soluble taxol derivatives. However, this kind of design has little value in targeted drug delivery.

Ester linkage was also used to produce camptothecin conjugate with a glycine derivative of maleimide-oligo (ethylene glycol) [122]. This pro-drug reacts with the cysteine-34 position of circulating albumin to form a conjugate, which is delivered inside the tumor cells by enhanced permeability and retention effect (EPR effect). Release of free camptothecin takes place inside the cells, after hydrolytic or dual hydrolytic and enzymatic cleavage of ester bond between camptothecin and glycine and the amide bond between glycine and the maleimide-oligo(ethylene glycol). In plasma, the half-life of the conjugates was between 9 and 15 hours.

Gemcitabine (GEM) conjugates with peripheral benzodiazepine receptors ligand connected through succinic acid moiety were used to target brain cancer [123, 124]. It was shown that prodrugs with ester type bond between GEM and succinic acid were unstable in serum, with half-life of about 5 minutes.

Ester-type linkage was used to connect paclitaxel to monoclonal antibody through C2'-hydroxy group using succinic acid residue as a linker [63]. This conjugate was much more active in the induction of apoptosis *in vitro* than paclitaxel itself. However, almost no differences in activity of monoclonal antibody-paclitaxel conjugates and naked monoclonal antibody could be detected *in vivo*. Low stability of the ester bond in circulation is a probable reason of this luck of specific activity.

Much better results were achieved for taxol connected to C2'-hydroxy group to the hyaluronic acid through an ester bound using succinic acid derivatives as a spacer [125, 126]. After receptor mediated internalization, free taxol was released from this conjugate by intracellular enzymatic cleavage of the labile C2' ester bond. *In vitro* data showed selective cytotoxicity of this pro-drug toward cell lines over expressing the CD44 receptor [125], which is the principal cell surface receptor for hyaluronate [127]. Unfortunately, this ester-based linker could also be hydrolyzed in hydrolase-free extracellural environment, with less then 20% of free taxol release during 24 h. In plasma environment, the hydrolysis was more significant [125].

Ester linkage was also used to connect doxorubicin to glutaric acid that was conjugated to the N-terminus of the cyclic peptide Cys-Asn-Gly-Arg-Cys (CNGRC) [39]. This type of ester bond was stable in circulation, with a half-life of 53 min. in human serum and 442 min. in the whole human blood.

In conclusion, ester linkages can be effective for some toxins that form exceptionally stable esters, such as paclitaxel. However, the general application of ester linkage for receptor-targeted drugs is limited by low stability in circulation.

Acid-Sensitive Hydrazone Linkers

In 1989, Silver and Haskel [128] described an acid-sensitive latent inhibitor of proteolytic enzymes, which was liberated in lysosomes by acidic hydrolysis of a hydrazone linker. The new linker was reliably stable at pH 7 and was cleaved at lysosomal pH 5. One year later, Greenfield and coworkers published two papers describing doxorubicin conjugated through the hydrazone linker to a monoclonal antibody [129, 130]. Doxorubicin hydrochloride was reacted with acylhydrazine forming acylhydrazone bond at the 13-keto position, and then the whole moiety was connected to the monoclonal antibody using a disulfide linker (Fig. **1A**).

Since then, various hydrazone linkers have been broadly used for many drugs, such as doxorubicin, morpholino-doxorubicin, streptomycin, 5-fluorouridine, chlorambucil, daunorubicin or vinblastine, as reviewed earlier [81, 84, 89, 101, 104].

The 4-maleimidophenylacetic acid hydrazone linker became the most common choice to connect doxorubicin or daunorubicin through the 13-keto position to a carrier peptide or monoclonal antibodies (Fig. **1B**). Recently, Krauss *et al.* [131] reported a new daunorubicin-carrier peptide conjugate that used this linker. Daunorubicin was connected to the 13-keto position through 4-maleimidophenylacetic acid hydrazone to a fragment of human calcitonin [C^8]-hCT(9-31). The pro-drug was stable at pH 7.4 and was able to release daunorubicin at pH 6. It was active *in vitro*, while daunorubicin, connected to [C^8]-hCT(9-31) peptide through a stable amide bond to the 3'-amino group from daunomycin sugar using 3-maleimidobenzoic acid as a linker moiety, remained inactive. Similar results were published earlier for daunorubicin conjugated through 4-maleimidophenylacetic acid hydrazone to the neuropeptide Y fragment [C^{15}]NPY [132]. The hydrazone was reasonably stable with less than 10% degradation occurring after 24 h at pH 7.4, while in cell medium, drug stability was found to be 90% after 48 h incubation. At pH 5, drug was released with an approximately 30 h half-life. The *in vitro* cytotoxic effect was tested on a neuroblastoma cell line expressing the NPY receptor and a glioblastoma line that did not. The conjugate with a hydrazone linker was as effective as daunorubicin against NPY receptor positive cell lines, and showed only marginal effect against cell lines without the receptor. In addition, daunomycin with an acid-stable linker but without a peptide attached was inactive.

A number of papers describing hydrazone acid-sensitive derivatives of albumin and transferrin conjugates with anthracyclines and chlorambucil has been published [133-138]. More recently, Kratz and coworkers have reported a linkage between doxorubicin and human serum albumin or transferrin, through phenylacetyl maleimide or alkalnoylhydrazone linkers [139-143]. It was proven that a simple maleimide phenylacetylhydrazone derivative of doxorubicin bound preferentially to endogenous serum albumin through an accessible Cys 34 upon incubation with whole human blood plasma or upon direct intravenous injection into mice [139]. The resulting conjugate showed superior antitumor properties against murine renal cell carcinoma. Both transferrin and albumin conjugates with doxorubicin incorporated through phenylacetyl hydrazone bond had significantly improved antitumor activities when compared to free albumin in the MDA-MB-435 breast carcinoma model. Identically constructed transferrin and albumin doxorubicin conjugates showed no distinct differences in antitumor effects [140]. Confocal microscopy demonstrated that the golgi and mitochondria were the predominant sites of accumulation for doxorubicin transferrin and albumin conjugates. For comparison, free doxorubicin was initially localized in the cell nucleus immediately after administration, and then translocated to the golgi and mitochondria [141]. In addition, doxorubicin hydrazone derivatives with aliphatic maleimide spacers forming *in vivo* conjugates with endogenous albumin also showed superior antitumor activities [143].

Hydrazone linker was also used to connect doxorubicin to the monoclonal antibodies through PHPMA polymer [120]. Free drug release was twice as efficient as from a pro-drug that was constructed with *cis*-aconityl linker. In general, pro-drugs with hydrazone linkages showed *in vitro* toxicity similar to free doxorubicin against EL4 lymphoma cell line and were much more efficient in releasing doxorubicin than their *cis*-aconityl analogues.

Acid-sensitive hydrazone linkers were used to connect drugs suach as paclitaxel or doxorubicin to the polyethylene glycol [144-146]. Paclitaxel and DOX conjugates with PEG20000 showed good stability at pH 7.4, with less then 10% free drug release after 48h, and reasonable cleavage time at pH 4-5 with half-lives ranging from 28h for Paclitaxel to 2h for DOX. They were active in *in vitro* experiments against many tumor cell lines, but also they showed a 2 to 40 fold decrease in activity when compared to the free drug [144, 145].

Hydrazone linker was used to build gentuzumab ozogamicin, which is a conjugate of P67.6 anti-CD33 antibody and calicheamicin. Gentuzumab ozogamicin was

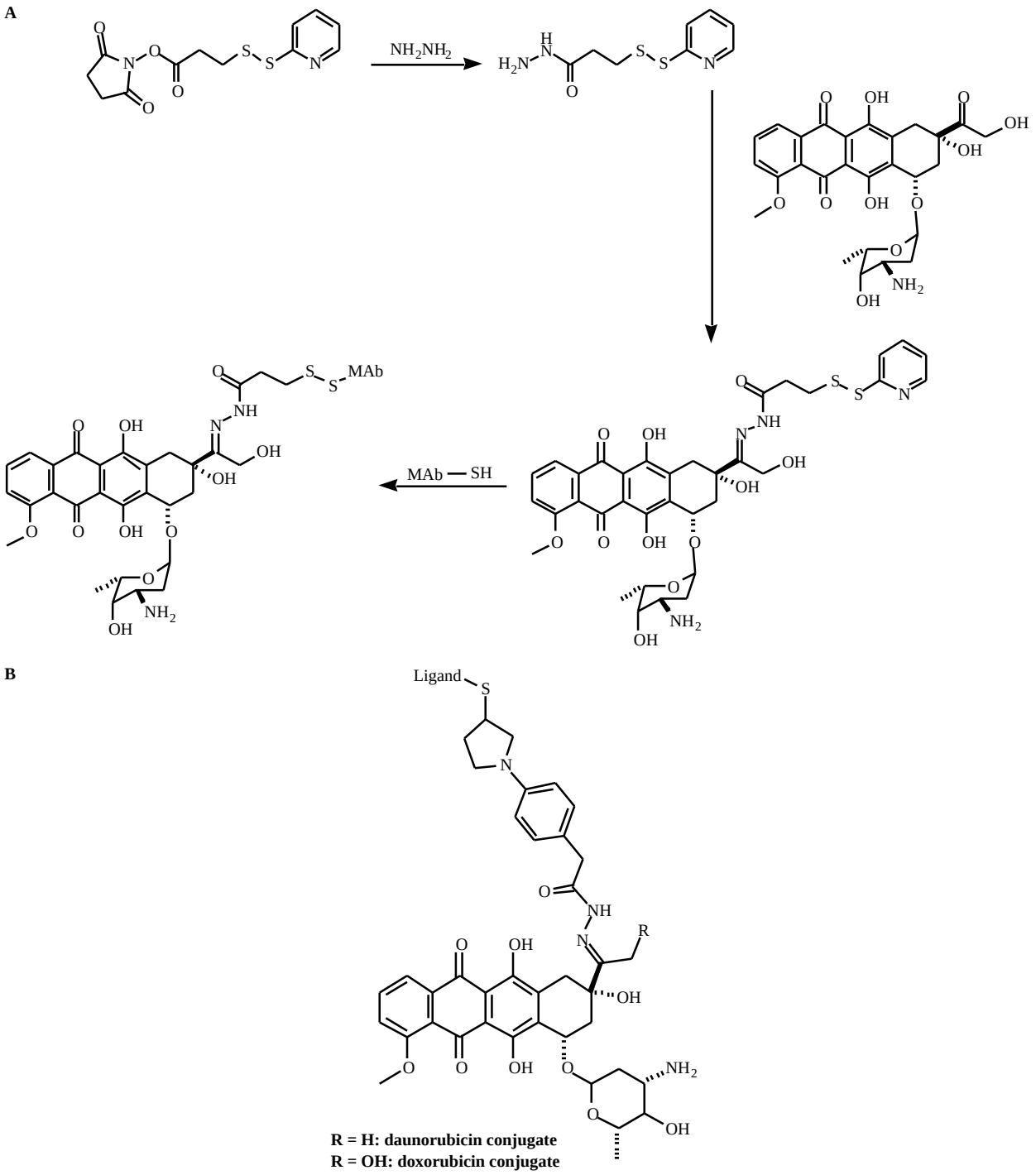


Fig. (1). Hydrazone linkers. **A**, Conjugation of doxorubicin to the monoclonal antibody (MAb) through acid-sensitive hydrazone linker [130]. **B**, Daunomycin or doxorubicin conjugates connected though the 13-keto position to the carrier peptides or monoclonal antibodies using 4-maleimidophenylacetic acid hydrazone linker [131].

designed for treatment of acute myeloid leukemia [147-158] and was approved for the treatment of patients with CD33 positive acute myeloid leukemia who were 60 years of age or older and are not candidates for cytotoxic chemotherapy [156]. This pro-drug is also in phase I clinical trials in pediatric CD33 positive acute lymphoblastic leukemia [152, 153]. The hydrazone linkage is critical for the activity of this pro-drug. Conjugate with the hydrazone linker was more than 7000-fold more potent then analogous amide conjugate in an *in vitro* experiment with CD33 positive HL-60 cell line. *In vitro* data also showed that the conjugate was 100-fold more cytotoxic then the calicheamicin [154]. On the

other hand, significant toxicity against CD33 negative Raji cells during long-term exposure was noticed. This can by explained by insufficient stability of hydrazone linker and slow intracellular release of N-Ac-gamma-calicheamicin [154]. In spite of the slow hydrolysis of the hydrazone linker in plasma, it appears to be the most effictive among acid-labile linkers and can be particularly useful for the attachment of the toxins with relatively low potency to the ligands of surface receptors that are expressed at relatively high densities on cancer cells.

Lysosomally Degradable Peptide Linkers

Immunotoxin conjugates that are internalized and delivered to lysosomes are exposed to many lysosomal proteases, mostly cathepsins and a number of exopeptidases. This linker family can be divided in two classes. The first class includes simple peptide linkers with specific sequences recognized by a lysosomal protease. In the second class, enzymatic cleavage triggers a reaction that causes self-elimination of the rest of the linker and toxin release.

Most of the lysosomally cleaved linkers are designed to be cleaved by either cathepsin B or D, since they are the most abundant lysosomal proteases. Cathepsin D is an aspartic protease that cleaves between two hydrophobic, preferably aromatic residues and requires at least a pentapeptide substrate for cleavage. Thorough characterization of cathepsin D (EC 3.4.23.5) specificity was conducted by Scarborough *et al.* [159, 160], and Peterson and Meares [161]. The best synthetic substrate contained p-nitrophenylalanine (Nph) in position P1', Phe in P1, Arg, Glu, Ser, Ala, Asp or Gln in P2 and Ile or Ala in P3. Other successful combinations (P3-P2-P1) included sequences Ile-Glu-Phe, Ile-Arg-Phe, Ile-Ser-Phe and Ala-Glu-Phe or Ala-Ala-Phe [160]. Peterson and Meares found preferential cleavage between two Phe residues (Lys-Pro-Ile-Leu-Phe-Phe(NO_2)-Arg-Leu and Phe-Ala-Ala-Phe(NO_2)-Phe-Val-Leu), preference for Thr in P3 position and distinct preference for Gly > Ala > Gln in P4. They did not observe preferences for Val, Ala and Asn at P2 position that had been predicted earlier [162]. Instead, Peterson and Meares suggested preferential cleavage of the substrates with hydrogen-bonding residues in P2 position. It should be noted, however, that the optimal residues in the secondary binding sites of the substrate depend on the nature of the side chains in both primary and other secondary binding sites. Thus, there is a whole array of good substrates for the enzyme that requires a relatively long peptide sequence for the cleavage and there is no contradiction in finding different optimums in a certain position for even slightly different sequences. Peterson and Meares have also analyzed cathepsin B (EC 3.4.22.1) substrate specificity. A hydrophobic residue (Val, Tyr(NO_2), Ala) was found to be preferred in position P2. P1 of a good substrate is occupied by an amino acid with a small side chain with the following order of efficiency Gly > Gln > Ala. Phe and Tyr residues are disfavored in this position. Asn, Phe and Tyr are underrepresented in positions P1-P4. Gln and Gly can also be favored in P4 position. Position P1' is occupied by the Phe residue. There is a strong preference for small residues such as Gly, Gln and Ala or 2-aminopropyl-1-yl-PEG in P3' position. Results obtained by Dubowchik *et al.* [163] have shown that in position P1, cathepsin B favored a basic amino acid that is protonated at pH 5, such as Lys. Citrulline, which forms only hydrogen bonds at this pH, is less desirable. Hydrophobic residues are preferred at the P2 site. If the P1 is occupied by Cit, significant differences in release rates are seen within a series of the following amino acids at P2: Trp > Ile > Phe > Leu > Val. In case of Lys in P1, there are no differences between Phe and Val in P2 position. Among the compounds tested, best hydrolysis was achieved when benzyloxycarbonyl (Z) group was present at P3 position. All tested compounds had *p*-aminobenzylcarbonyl (PABC) spacer at P1'. Barrett [164] reported that Z-Phe-Arg-X (where X is a fluorogenic moiety) is a common substrate for cathepsin B and L.

Hopewell, Duncan *et al.* described preclinical cardiotoxicity evaluation of a conjugate of a N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer with doxorubicin-galactosamine (PK2) [165]. In this drug, cathepsin B, H and L sensitive Gly-Phe-Leu-Gly linker was used [166-169]. This new design aimed to promote liver targeting by the galactosamine residue connected to the enzyme-sensitive linker. The galactose residue provides for a selective capture by the asialoglycoprotein receptor of hepatocytes and hepatocellular carcinoma [165, 170, 171]. PK2 conjugate allowed the use of doxorubicin (DOX) doses that were increased about five times over free DOX in DMF, without detectable cardiotoxicity. Schatzlein *et al.* used the same peptide sequence Gly-Phe-Leu-Gly to conjugate DOX with cyclic nanopeptide that targeted the transferrin receptor. The cyclic nanopeptide was chosen from a phage display library. The final structure of the cyclic nanopeptide was not disclosed [172].

Another enzyme cleavable peptide linker, Ala-Leu-Ala-Leu-Ala was used to connect ellipticine derivative 1-[3-[N-(3-aminopropyl)-N-methylamino]propyl]amino-9-methoxy-5,11-dimethyl-6H-pyrido[4,3-b]carbazole (Ell) to the heptagastrin peptide (5-Nle) (7G) [173]. Ellipticine amino group was connected to the ALALA C-terminus and this conjugate was coupled to gastrin N-terminus through succinic acid (Suc). In addition, ellipticine conjugates with 7G and human gastrin I (2-17) (15-Nle) (HG) with carbamate linkage was synthesized. Both conjugates with carbamate linkage showed poor cleavage (less then 20% in 2h) by cathepsin B and D. In comparison, 90% of ellipticine linked through ALALA to the heptagastrin peptide was cleaved by cathepsin D. About 70% of the cleavage occurred in the linker part of this compound, mostly in the following positions: -Suc-Ala-Leu-Ala-Leu-Ala-Ell and -Suc-Ala-Leu_Ala-Leu-Ala-Ell. Cathepsin B cleaved substrate in ≈50% yield, but mainly in the gastrin portion of the construct between Gly^5 and Trp^4 (-Suc-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2) [173]. The same linker and its shorter derivative Leu-Ala-Leu-Ala were also used to connect ellipticine to the VIP [174]. Ellipticine was connected to the C-terminal carboxyl group of ALALA and LALA, while these derivatives were connected directly to the C-terminal carboxyl of VIP. Both conjugates showed potency against breast cancer cell lines (MCF-7), but VIP-LALA-Ell was a little more potent, probably because of better binding affinity to the breast cancer cells as compared with VIP-ALALA-Ell. A similar peptide, Ala-Leu-Ala-Leu was previously successfully used for daunomycin conjugates [175, 176].

The same peptide sequence, but containing a β-alanine was used by Fernandez *et al.* to prepared *N*-succinyl-(β-alanyl-L-leucyl-L-alanyl-L-leucyl)doxorubicin as a low toxic extracellularly tumor-activated prodrug [177, 178].

Simple D-Val-Leu-Lys peptide was used as a linker specific for plasmin activation of cytarabine from α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) polymer [179]. PHEA-D-Val-Leu-Lys-cytarabine was very stable in plasma, but it was able to release the drug in the presence of plasmin within 24 h with the half-life ~ 6 h.

Another peptide linker, Gly-Phe-Leu-Gly-Leu has been shown to release the glycine derivative of 5-fluorouracil (Gly-α(5-FU); 2-(5-fluorouracil-1-yl)-L-glycine) from N-(2-hydroxypropyl)methacrylamide-based copolymers (HPMA) via cleavage by lysosomal enzyme cathepsin B followed by tritosome aminopeptidase cleavage [180]. Compounds with shorter linkers, such as HPMA-Gly-Phe-Ala-Gly-α(5-FU) or HPMA-Gly-Phe-Leu-Gly-α(5-FU) were not cleaved by cathepsin B at all. Pentapeptide containing derivatives HPMA-Gly-Phe-Leu-Gly-Ala-Gly-α(5-FU) and HPMA-Gly-Phe-Leu-Gly-Leu-Gly-α(5-FU) were cleaved by cathepsin B, but only Leu-Gly-α(5-FU) intermediate could be converted to free Gly-α(5-FU) by tritosome aminopeptidase. In addition, only one diastereoisomer L-Leu-L-Gly-α(5-FU) could be converted to free Gly-α(5-FU) while the other, L-Leu-D-Gly-α(5-FU) remained uncleaved. Similar strategy, utilizing HMPA spacer and Gly-D,L-Phe-Leu-Gly linker was successfully used by Kovár *et al.* [181] to prepare doxorubicin targeted to the transferrin receptor.

In many cases, a part of the linker is still attached to the drug molecule after an initial cleavage by an endopeptidase. Exopeptidases such as dipeptidyl peptidase I (cathepsin C, EC 3.4.14.1), or lysosomal cysteine peptidase with a broad range of activity [182] can process the prodrug even further.

An interesting observation was made by Roseeuw *et al.* [183] during the development of polymeric prodrugs of antibiotics. Gly-Phe-Leu-Gly and Gly-Phe-Ala-Leu [180], linkers were used as cathepsin B substrates for the construction of dextran-norfloxacin antibiotic conjugate. A pro-drug with norfloxacin incorporated via tetrapeptide spacer as an α-C substituent of the terminal glycine residue released the parent drug after cathepsin B cleavage. In the prodrug, the piperazine ring of norfloxacin substituted for a hydrogen atom of the methylene group of the glycine residue. The substituted glycine is unstable unless its amino group is engaged in amide bond formation. Enzymatic cleavage of the amide bond causes the release of the secondary amine bond in the α-position of the glycine. Thus, the approach appears to be a useful tool for the construction of another type of traceless conjugate for targeted cancer therapy.

Even a simple spacer Gly-Gly-Gly turned out to be useful as a lysosomally degradable linker [184-187]. It was used to connect camptothecin analogue (7-ethyl-10-aminopropyloxy-camptothecin) to the carboxymethyl dextran polymer. The compound showed slow, steady release of the camptothecin derivative, and was active against MX-1 human mammary xenograft tumors. Linkers with one or two glycines were almost resistant to enzymatic cleavage, while those with four or five Gly residues were cleaved much faster then triglycine spacer. Some poly(α-amino acids) copolymers based on poly(L-glutamic acid) have also been applied as spacers degradable in lysosomes [188].

Kratz and coworkers used a simple amide bond between methotrexate (MTX) α or γ carboxyl group and aminopolyethylene glycol, as a lysosomally degradable linker [189]. Methotrexate was connected to aminopolyethylene glycol (MW 5000, 20000 and 40000) using dicyclohexylcarbodiimide (DCC) chemistry. The resulting MTX-PEG conjugates consisted of mixtures of α- and γ-isomers and L- and D-enantiomers. The most active compound, MTX-PEG40000 showed superior efficacy compared to free MTX. It was stable in PBS buffer, but a significant amount of MTX was cleaved from the conjugate in a cell-conditioned medium or human serum, probably because of enzymatic cleavage by enzymes, such as γ-glutamyl hydrolase.

Vega *et al.* [62] described targeting doxorubicin to epidermal growth factor by conjugation of monoclonal antibody C225 to poly(L-glutamic acid) through a polyethylene glycol spacer. Previously described conjugates of poly(L-glutamic acid) polymer and camptothecin [190-192] were delivered to cancer cells not by receptor-mediated pathways but through the aforementioned EPR effect [193-198]. However, Oldham *et al.* [199] believe that poly(L-glutamic acid)-paclitaxel conjugate does not enter the cells *in vitro*, but rather the free drug is transported into the cells after slow release of paclitaxel from the conjugate. Doxorubicin was reported to be released from C225 monoclonal antibody conjugate after cellular internalization, but the authors did not provide any details about this process. There is only a short note that the conjugate was stable in rabbit plasma and was biodegradable through cleavage of poly-(L-glutamic acid) backbone [62]. The later papers also did not specify how drug was released from this kind of polymer [190-192,199]. It is only known that cathepsin B is capable of cleaving paclitaxel from poly-(L-glutamic acid) with generation of paclitaxel-deglutanates[2]. It is unknown which enzyme is responsible for cleaving the paclitaxel-diglutamate to paclitaxel-glutamate, which is unstable and breaks down to paclitaxel and glutamic acid [200].

Hydrolase-Dependent Self-Eliminating Linkers

This type of spacers has an advantage of generating non-modified toxins and thus is less likely to compromise the intrinsic activity of the delivered drug. Two types of self-eliminating linkers have been described to date, cyclization spacers and 1,4 or 1,6-elimination spacers. Papot *et al.* [201] have summarized the general chemistry of both spacer types and a broader application to selectively activated drugs in a recent in-depth review. De Groot et al [202] have compared both types of self-eliminating spacers on paclitaxel derivatives activated by plasmin cleavage of D-Val-Leu-Lys tripeptide. Although the system utilizes an extracellular protease, an analogous approach with appropriate modification in the

[2] Shaffer SA, Baker Lee C, Nudelman E, Kumar A, Coon M, Stone I, De Vries P, and Singer JW. Metabolism of poly-L-glutamic acid (PG) paclitaxel (CT-2103); proteolysis by lysosomal cathepsin B and identification of intermediate metabolites. Proc Amer Assoc Cancer Res 2002; 43: 416.

cleavable peptide can be adapted for the lysosomal activation. Compounds **2**-**5** (Fig. **2A**) were designed with a variety of *N,N'*-substituted ethylenediamine derivatives that undergo a ring closure upon peptide cleavage with the release of the free drug (Fig. **2A**). Compound **2** was unstable in a buffer solution at pH 7.3 because paclitaxel residue decomposed spontaneously by internal elimination to baccatin III. The plasmin was unable to cleave prodrugs **3** and **4**, probably because of the steric hindrance imposed by the methyl-substituted amide bond on the enzyme active site. The compound **5** with methyl substituent on carbamate nitrogen and hydrogen on amine nitrogen ($R_4 = CH_3$, $R_4 = H$; Fig. **2A**) was stable for undesired reaction yielding baccatin III at pH 7.3, and was easy hydrolyzed by plasmin to yield paclitaxel. However, the cyclization was very slow with the half-life of prodrug **5** spacer being 23 h. In comparison, the conjugate **1** with 1,6-elimination spacer generated paclitaxel instantaneously upon enzymatic cleavage. Although not all the possibilities with the ring closure eliminations have been exploited so far, it appears that 1,4 and 1,6-eliminations occur much faster, proceed much more efficiently and consequently have found much broader applications.

1,6-elimination in benzylic systems was originally suggested as a means of prodrug activation by Katzenellenbogen *et al.* in 1981 [203]. The process is triggered by the generation of an electron donating amino or hydroxyl group from a less electronegative protected amino or hydroxyl functionality (Fig. **3A**). Dubowchik *et al.* [163, 204, 205] have studied the efficacy of the drug regeneration in a model system that consisted of cathepsin B-sensitive dipeptide and a self-immolative *p*-aminobenzylcarbonyl (PABC) spacer connected to doxorubicin. Half-life of Z-Phe-Lys-PABC-DOX (Fig. **3B**) at 37°C was 8 min upon the treatment with cathepsin B. The compound was not hydrolyzed in human plasma under the same conditions over a 6-7 h time period. The authors have also described several other di and tripeptide spacers. The best plasma stability along with fast cathepsin B-activated release were found in Z-Phe-Lys-PABC-DOX, Z-Val-Lys-PABC-DOX, D-Phe-Phe-Lys-PABC-DOX, D-Ala-Phe-Lys-PABC-DOX, Ac-Phe-Lys-PABC-DOX, HCO-Phe-Lys-PABC-DOX and Z-Val-Lys-PABC-DOX derivatives [163]. These linkers have been used to connect DOX to the chimeric monoclonal antibody BR96 that binds an antigen related to Lewis Y abundantly

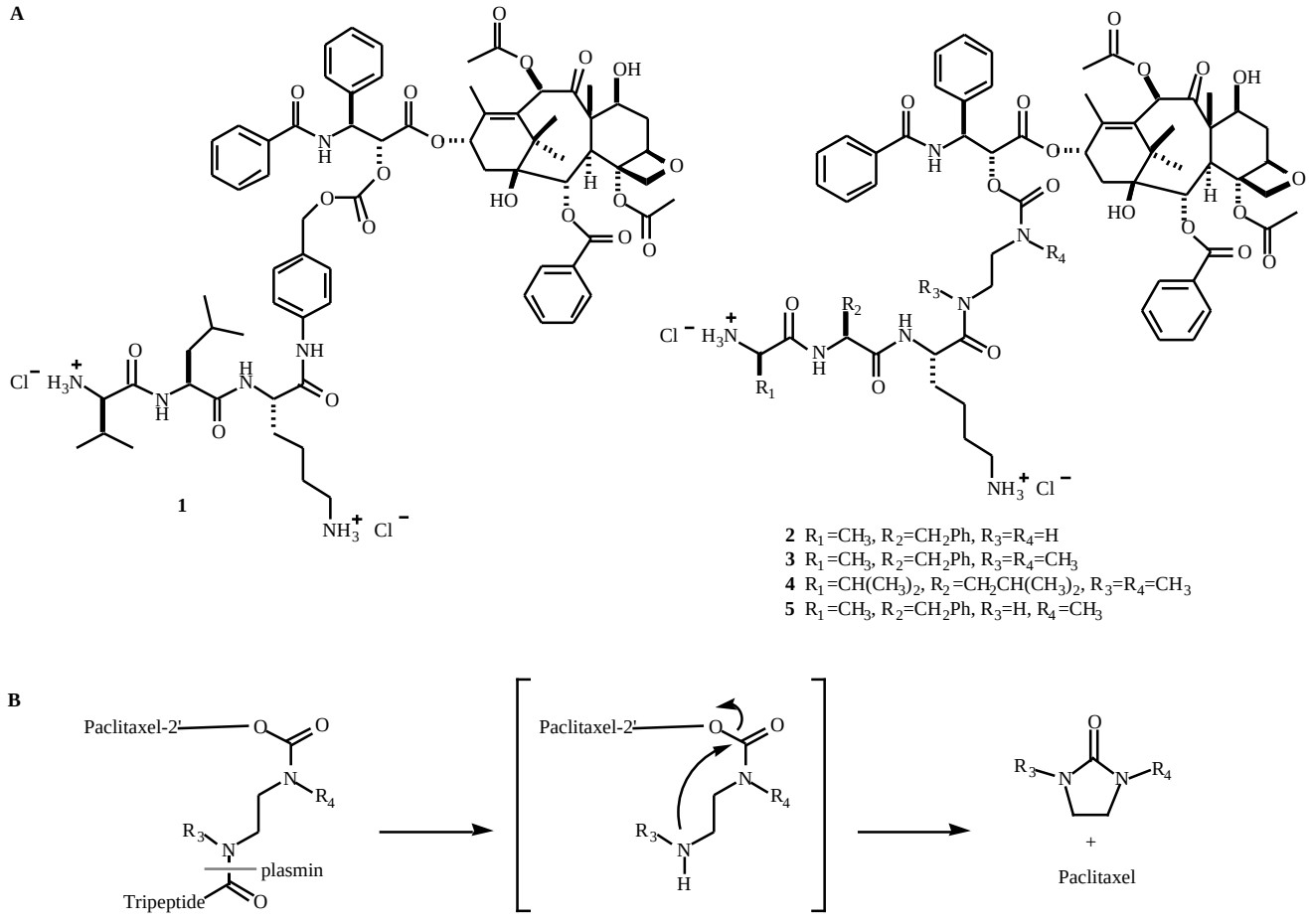


Fig. (2). Bivalent enzyme cleavable linkers applied for conjugation of camptothecin to monoclonal antibodies. **A,** 1,6-Elimination spacer (1) and *N,N'*-substituted ethylenediamine derivatives (2-5) that undergo a ring closure upon peptide cleavage [202] . **B,** Self-degradation of *N,N'*-substituted ethylenediamine spacer after enzymatic cleavage of the tripeptide.

expressed on the surface of cells from many human carcinomas [205]. Antibodies were conjugated to the linker through succinic acid to generate two conjugates, BR96-Suc-Phe-Lys-PABC-DOX and BR96-Suc-Val-Cit-PABC-DOX. Both conjugates showed potent antigen-specific cytotoxic activity against tumor lines with varying level of BR96 expression (lung adenocarcinoma L2987, ovarian carcinoma A2780, colorectal tumor HCT116) and remarkable selectivity when compared to the non-binding conjugates [205].

Almost the same linkage was used by de Groot *et al*. [206] to connect bicyclic CDCRGDCFC (RGD-4C) peptide that selectively binds $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, to the DOX. In this particular case, –Suc-D-Ala-Phe-Lys-PABC- linker was used. D-Ala-Phe-Lys-PABAC spacer was previously extensively investigated by de Groot and coworkers with doxorubicin, daunorubicin and 9-aminocamptothecin and showed excellent stability and specific activation [207, 208]. The prodrug RGD-4C-Suc-D-Ala-Phe-Lys-PABC-DOX was designed to be plasmin activated, but only 30% of free doxorubicin was released upon incubation with the enzyme probably because of the poor solubility of the compound. The prodrug was tested against HUVEC and HT1080 cells and in the presence of 15μg/ml plasmin showed almost the same toxicity as free doxorubicin.

Recently, Val-Cit-PABC linker was used to conjugate a derivative of tubulin binding toxin, monomethyl auristatin E (MMAE) to a chimeric monoclonal antibody cAC10, directed against CD30. Val-Cit-PABC-MMAE was attached to the cAC10 through stable thioether bond, which was formed by controlled partial reduction of internal cAC10 disulfides with DTT, followed by addition of the maleimide-Val-Cit-PABC-MMAE [209]. cAC10-Val-Cit-PABC-MMAE conjugate showed good stability in human plasma, with the half-life exceeding 250 days. It turned out to be a potent and selective toxin for the cells expressing CD30, such as Karpas 299, SU-DHL-1 or HDML-2. However, no direct correlation was found between antigen expression level and sensitivity. Although CD30 expression was required for activity, SU-DHL-1 cells with lower binding ratio than HDML-2 were about 1000-fold more sensitive to cAC10-Val-Cit-PABC-MMAE prodrug. The same cells showed equal sensitivity to free MMAE suggesting that other factors (such as the rate of internalization or enzymatic cleavage in lysosomes) may contribute to the sensitivity.

Walker at al. [210] synthesized an immunoconjugate of monoclonal antibody BR96 and camptothecin (CPT) linked through Phe-Lys-PABC moiety. A maleimide group was used as a spacer between BR96 and Phe-Lys-PABC-CPT to ensure the accessibility of that dipeptide bond to the enzyme. Monoclonal antibody was connected through a stable thioether bond, formed via reduced cysteine (Fig. **3C**). Synthesized prodrug showed cytotoxic activity against L2987 cell line, and was 30 times more active than non-tumor binding conjugate with immunoglobulin G. In addition, cBR96-camptothecin conjugate was at least as potent as the free drug with IC_{50} [CPT] for conjugate equal to 0.1 μM and for free CPT 0.4 μM, respectively.

Higher toxin load was achieved recently by incorporation of Phe-Lys-PABC linker with bivalent maleimide spacers [211, 212]. One such spacer, {carboxymethyl-[2-(2,5-dioxo-2,5-dihydro-pyrrolo-1-yl)-ethyl]-amino}-acetic acid was connected to the BR96 monoclonal antibody and coupled through two Phe-Lys-PABC linkers with two molecules of DOX [212]. This conjugate showed selective anticancer activity against L2987 cell lines, with IC_{50} = 0.2 μM (calculated for DOX concentration), while similar conjugate with IgG was much less active IC_{50} = 5.0 μM (calculated for DOX concentration). These immunoconjugates exist almost exclusively as non-covalent dimmers, due to the hydrophobicity of the linkers and of doxorubicin itself. Aggregation did not appear to impair their *in vitro* activity, however, these non-covalent dimers can be recognized *in vivo* as foreign bodies and cleared by the reticuloendothelial system [213]. To prevent possible problems with conjugate aggregation, additional methoxytriethylene glycol (mTEG) chain was attached to doxorubicin via a hydrazone bond [146]. Both of the immunoconjugates showed specific immunotoxicity against human lung carcinoma L2987 (IC_{50} = 0.1 (μM DOX) for Val derivative and IC_{50} = 0.2 (μM DOX) for Phe) and also high immunoselectivity, at least 50-100 folds more, when compared to respective IgG controls. It was proven that hydrophilic mTEG chain prevents nonspecific aggregation, and its hydrazone linkage is hydrolyzed in lysosoms due to lower pH (~5) while remaining stable in circulation (pH = 7).

de Groot *et al*. attempted to use multiple PABC or a combination of PABC with bisamine cyclization spacers [214]. H-D-Ala-Phe-Lys-$(PABC)_2$-DOX (**a**), H-D-Ala-Trp-Lys-$(PABC)_2$-DOX (**b**), H-D-Ala-Phe-Lys-$(PABC)_3$-DOX (**c**), H-D-Ala-Phe-Lys-$(PABC)_2$-paclitaxel (**d**) and H-D-Ala-Phe-Lys -PABC-*N,N'* - dimethylethylenediaminecarbamate - paclitaxel (**e**) were compared to the conjugates containing only single PABC or bisamine cyclization spacer system. All new prodrugs were stable in 0.1 Tris/HCl buffer (pH 7.3) for 3 days at 37 °C with no signs of degradation. Paclitaxel derivatives **d** and **e** showed a decrease of *in vitro* cytotoxicity when tested in a panel of seven cell lines, of respectively 3.7-fold and 73-fold in comparison with paclitaxel itself. Doxorubicin prodrugs **a**, **b** and **c** were also less cytotoxic with respectively 30-fold, 24-fold and 22-fold reduction in activity. All multiple PABC spacer systems showed significantly higher plasmin activation rate then prodrugs containing only one PABC spacer. Activation rate was 2 to 3-fold higher for doxorubicin prodrugs and 6-fold higher for paclitaxel multiple PABC prodrug. The disadvantage of multiple spacers, however, is in their higher hydrophobicity and consequently lower solubility of the resulting conjugates.

Prodrugs with a combination of PABC spacer and bisamine cyclization spacer (**e**) were cleaved 10-fold faster with plasmin but had reduced cytotoxicity when compared to a prodrug containing only PABC spacer, which correlates with generally slower rates of release from cyclization spacers.

de Groot *et al.* [214] have also tested spacer systems that rely on 1,8- or 1,10-elimination for the drug release (Fig. **4**). Both naphthalene and biphenyl spacer system were resistant to the expected 1,8- and 1,10-elimination reaction, probably due to the higher energetic barrier for the process, which requires the aromaticity from two aromatic rings to be sacrificed. In addition, for 1,10-elimination to occur, biphenyl ring system has to reach a planar conformation, which is energetically highly unfavored.

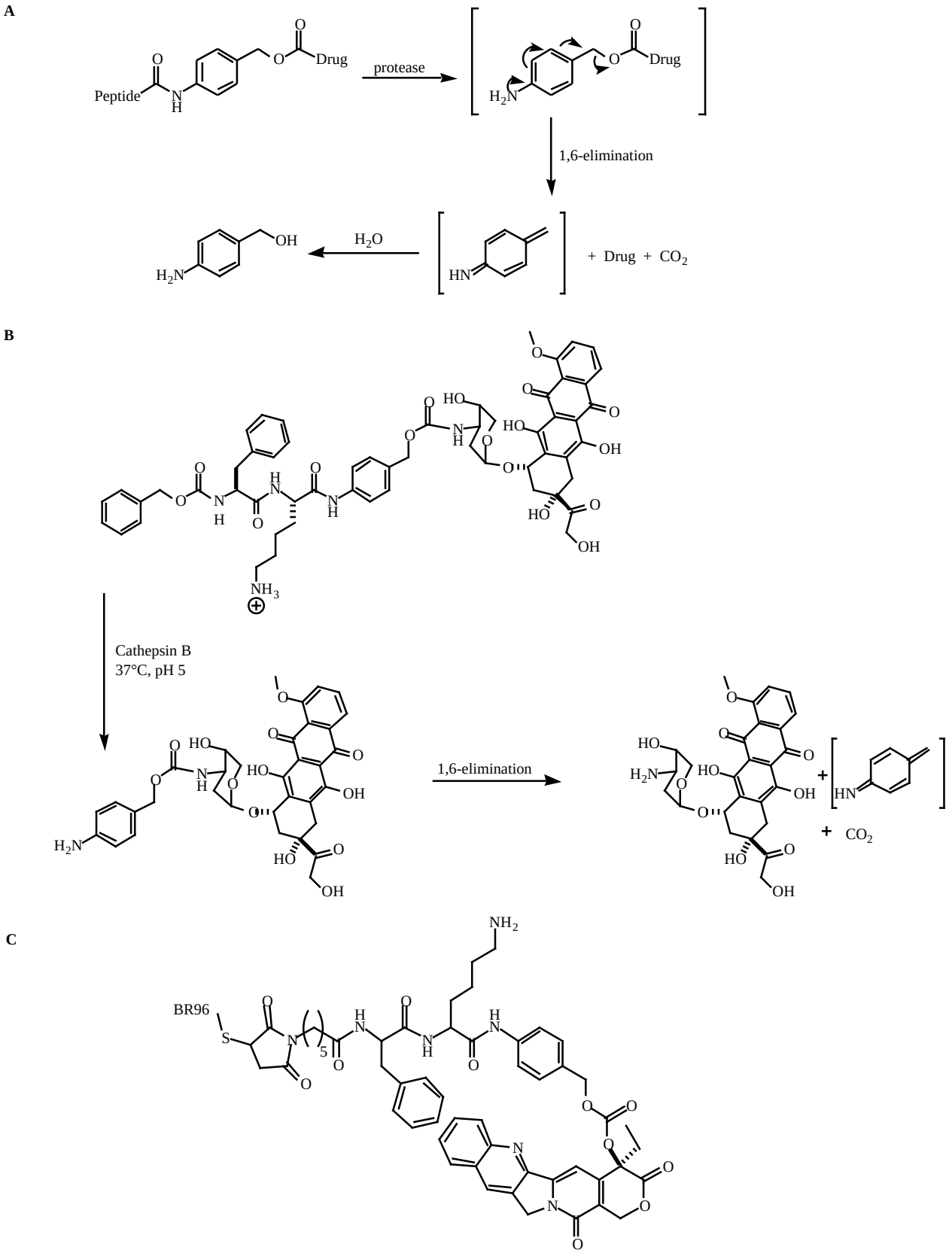


Fig. (3). Conjugates with 1,6-elimination spacers. **A**, Mechanism of 1,6-elimination. **B**, Z-Phe-Lys-PABC-DOX conjugate [163]. **C**, Camptothecin conjugate with BR96 monoclonal antibody connected through a bivalent cathepsin B cleavable linker [210].

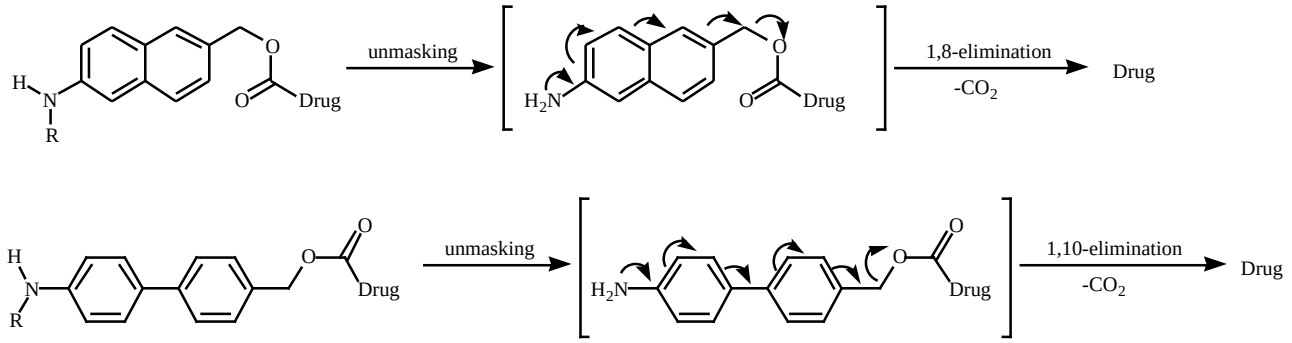


Fig. (4). 1,8- and 1,10-eliminations spacers [214].

p-Aminobenzyl carbamate derivatives are well suited for the attachment of drugs containing free amino groups because the amide bond formed during the coupling to the carboxyl group of the linker is fairly stable. In contrast, the carbonates formed during the attachment of the drugs with hydroxyl groups are usually hydrolytically unstable. To generate a self-immolative spacer that would form stable derivatives of toxins with hydroxyl groups, Toki *et al.* have tested 1,6-elimination of *p*-aminobenzyl ethers (PABE) (Fig. **5A**) [215]. Two new model prodrugs with cathepsin B-sensitive Z-Val-Cit-PABE linker connected to a topoisomerase inhibitor etoposide (**A**) and a tubulin-binding agent combretastatin A-4 (**B**) have been synthesized (Fig. **5B**). Both conjugates were stable in buffers for at least 7 days at pH 5.1 and 7.2. Compound **A** turned out to be unstable in human serum with the half-life of 48 h because of etoposide moiety decomposition, whereas compound **B** remained unchanged for more than 7 days. Cytotoxicity was studied *in vitro* on human lung adenocarcinoma L2987, human melanomas WM266/4 and IGR-39. Conjugate **A** was 20 to 50 times less active then etoposide, and conjugate **B** showed 13-fold less activity against L2987 cell line then combretastatin A-4. In the same assay, combretastatin A-4 conjugate with classic PABC linker Z-Val-Cit-PABC- combretastatin A-4 was as cytotoxic as the toxin itself. Thus, the new PABE linker is more stable than PABC linkage. It has been proven, that both **A** and **B** conjugates released the toxins by specific activation by cathepsin B followed by 1,6-elimination of PABE moiety. Specific activity of cathepsin B, measured as the lost of starting material at 37 °C in pH 5.1 acetate buffer, was 160 (nmol/min/mg) for **A** and 61 (nmol/min/mg) for **B**. Consequently, the second step must have been responsible for the apparent differences in the rates of toxins release.

Sulphydryl Linkers

The disulfide bond can by reduced in circulation, in the cytosolic space, in the endoplasmic reticulum, at the cell surface, in endosomes and lysosomes. A high difference in a redox potential between extracellular oxidizing and intracellular reducing conditions makes the disulfide bond a promising linker, which should be fairly stable in circulation and easily cleavable after cellular uptake. The use of disulfide linkers for drug delivery and especially the mechanisms of disulfide bond reduction have been recently reviewed [216]. In addition, earlier papers addressing the usage of disulfide bonds in the context of drug delivery were discussed in the earlier reviews [5, 81, 89].

Recently, Tochler *et al.* [69] published the results of successful phase I clinical trials of cantuzumab mertansine used for treating patients with CanAg-expressing solid malignancies . This tumor-activated prodrug is a derivative of maytansine connected to a humanized monoclonal antibody huC242 through a disulfide linker. Cantuzumab mertansine is cleaved intracellularly and in plasma by disulfide linkage reduction. The half-life in plasma is 41.1 h.

New series of potent second-generation taxoids, connected by disulfide linker to the monoclonal antibodies, were synthesized by Ojima *et al.* [217]. Taxol derivative was modified at C-10 position by methyldisulfanyl alkanoyl group, which was used later to connect this new taxoid derivative to the monoclonal antibody. Other taxoid positions such as C7, C2 or C2' were also investigated. It was shown, that modification at the C2' position produced much less potent taxoid derivatives. Modification by 3-methyldisulfanylbenzoyl moiety at C7 and C10 position resulted in substantial loss of activity. Free taxoid was released after internalization and disulfide bond cleavage. *In vivo* experiments showed a complete growth inhibition of A-431 tumor xenograft in SCID mice. No toxicity was detected at therapeutic concentration, as demonstrated by the absence of weight loss.

In this same year, another research group investigated a water soluble form of paclitaxel activated by reductive cleavage of the disulfide bond [218]. In this case, paclitaxel was connected by an ester bond at C2' position to the 2,2-dimethyl-4-mercaptobutyric acid. The resulting modified taxol molecule was connected to different hydrophilic molecules by disulfide bond formation. Best results were obtained for captopril derivative, which showed much better therapeutic properties then free taxol against L2987 lung carcinoma implanted into athymic nude mice. However, it is possible, that anti-angiogenic effects of captopril combined with paclitaxel cytotoxicity, contributed to the superior properties of the conjugate.

Captopril was also used by Kok *et al.* [219] to make a prodrug targeting the kidney. Captopril was connected to the lysozyme lysine sidechain by 4-(1-mercaptoethyl)benzoic acid spacer. This pro-drug was not receptor targeted, and delivery was based on accumulation of low-molecular-

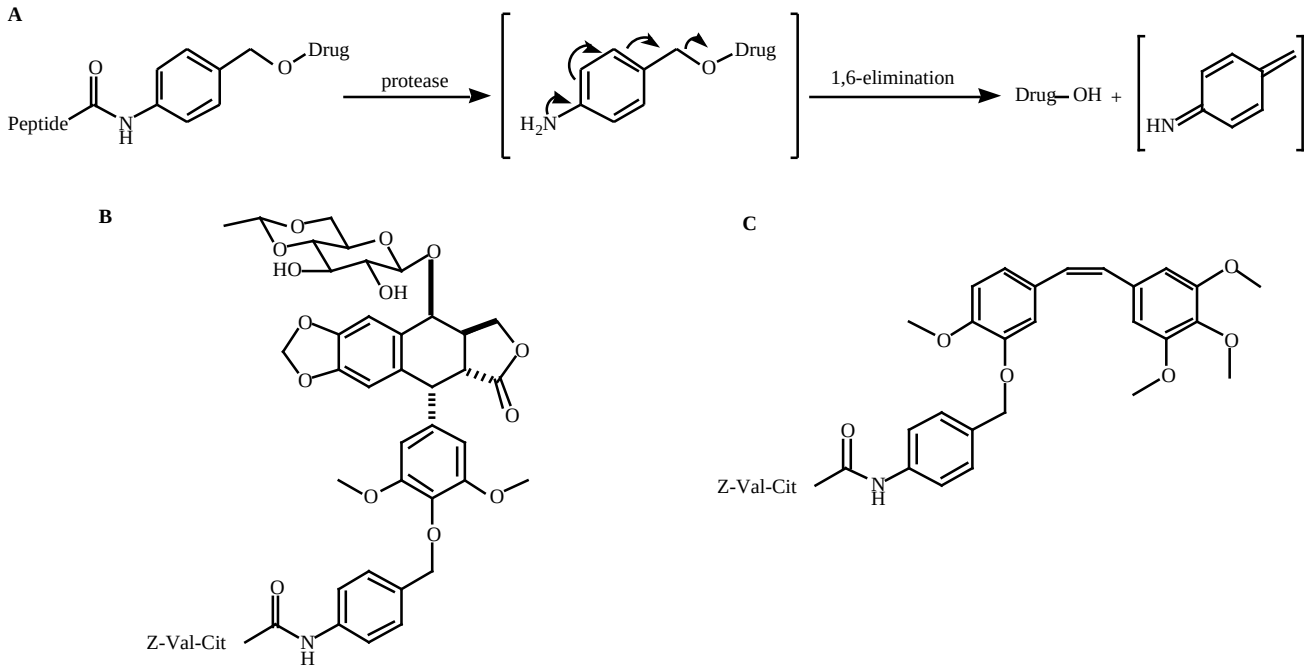


Fig. (5). 1,6-Elimination of *p*-aminobenzyl ethers [215] (A). Conjugates of etoposide (**B**) and combretastatin A-4 (**C**) using *p*-aminobenzyl ether linker strategy

weight proteins in the proximal tubule cells of the kidney. However, this disulfide bond can by useful for other kinds of conjugates. It was showed, that cleavage appears in the presence of high concentration of glutathione in the kidney proximal tubule cells.

Other Linkers

Very interesting, light sensitive linker was used by Gilbert *et al.* [220] to make the prodrug A-Z-CINN 310 which consist of paclitaxel, A-Z-CINN™ linker and trastuzumab monoclonal antibody. The photochemical properties and the structure of pH sensitive linker A-Z-CINN™ (Fig. **6**) were not disclosed in the paper, but are available from two US patent applications [221, 222]. A-Z-CINN™ linker structure is based on a modified cinnamic acid, and the linker is bound to the paclitaxel at C2' position, by forming an ester bound. Very similar light sensitive linker was earlier developed by Porter and Bruhnke [223, 224] to form light sensitive acyl-enzymes.

A-Z-CINN 310 prodrug, with paclitaxel loading of 0.023mg per 1mg of monoclonal antibody, showed superior properties against HER-2-positive breast tumor cells

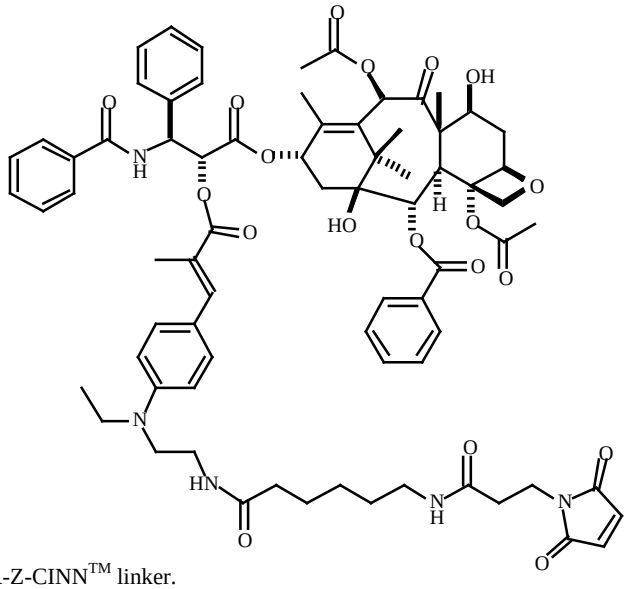


Fig. (6). Paclitaxel conjugate with A-Z-CINN™ linker.

implanted in mice, especially when drug release was light accelerated [220]. Ad advantage of A-Z-CINN™ linker is that it can be used to connect any targeting molecule with any drug, using appropriate chemistry.

Another interesting attempt to provide a new way of pro-drug activation in hypoxic tumor tissue, was made by Scheeren and coworkers [225]. Two bioreductive activated pro-drugs have been proposed: 2'-(4-nitrobenzyl carbonate) paclitaxel and 3'*N*-(4-azidobenzoylcarbonyl)-3'*N*-debenzoyl-2'*O*-benzoyl-paclitaxel. Bioreduction of the nitro group from 2'-(4-nitrobenzyl carbonate) paclitaxel caused 1,8-self-elimination of the 4-amino- or 4-(*N*-hydroxyamino)benzyl moiety to give the parent drug. The second pro-drug is activated in a different way. After azide reduction and 1,4-self-elimination, a free amine is formed at C3' position. Migration of the benzoyl at C2' to the C3'-NH_2 causes the formation of paclitaxel. Although both linkers have not been tested in receptor-mediated drug delivery, it might be possible to modify them in the future and to introduce an additional attachment point for receptor-targeting molecule.

TOXINS

The choice of a toxin for use in receptor-mediated drug delivery must meet a number of stringent criteria. First, the inherent toxicity must be very high because the expression of the receptors rarely exceeds 10^5 molecules per cell. We have estimated previously that the maximum concentration of the drug delivered through receptor-mediated endocytosis cannot exceed 10^{-7} M, even if all processes occur with 100% efficiency. Thus, in order for the warhead to be effective, its toxicity should be in the low nanomolar range [174]. Our early work on delivery of various alkylating agents, such as triazenes, melphalan and chlorambucil into gastrin receptor-positive cells required high concentrations of the conjugated toxins, in spite of the fact that there was evidence of excellent differential toxicity [226]. Second, the delivery of the toxin requires that the receptor ligand, modified by the linker and the toxin be processed in lysosomes. Consequently, the toxin itself must be able to survive the proteolytic processing and the acidic environment of the lysosome. Moreover, it has to be able to leave the lysosomal compartment once the processing has occurred. For example, polyglutamated methotrexate attached to the heptagastrin via the ALALA linker was efficiently taken up by NIH3T3-GR cells, and was deposited into lysosomes, where it stayed without detectable diffusion to cytoplasm [227]. Presumably, the multiple negative charges prevented the toxin from penetrating the lysosomal membrane. A third problem is that some drugs have very efficient uptake mechanisms of their own. For example, doxorubicin conjugated to heptagastrin, showed no differential toxicity between GR+ and GR- cells. However, this phenomenon appears to be dependent on the ligand. Daunomycin (closely related to doxorubicin) attached to melanotropin exhibited receptor-mediated toxicity in murine melanoma cells [228]. Fourth, even toxins that have the reputation for non-selective toxicity, are not necessarily equally toxic in all cell types. This can be a disadvantage if one wants to target the tumor in which the toxicity is low, but it can be a distinct advantage when the expression of the receptor is high at more than one site. Thus, ellipticine conjugated with VIP whose receptor is over-expressed in small cell lung cancer and in breast cancer [14], was highly selective but not very potent in MCF-7 breast cancer cells [175]. The ellipticine-heptagastrin conjugate had sub-nanomolar activity in NIH3T3-GR cells and in xenografts in nude mice derived from these cells [174]. However, there was no toxicity in kidneys, where the expression of GR is very high.

There are two other very important characteristics of the toxin that must be kept in mind when designing a receptor-targeted drug. Firstly, the chemistry of conjugation to the linker and the ligand has to be straightforward. The toxin-linker-ligand construct is invariably a complex molecule, with many potential sites for chemical reactions. Thus, the chemistry has to be compatible with the rest of the molecule, without the need for special protecting groups beyond those that are required to assemble the required peptide sequence. Secondly, the toxin has to be freed from the linker and the ligand in an active form. Lysosomal processing will frequently leave a part of a linker on the toxin. It is thus important to utilize toxins where the presence of these unhydrolyzed residues will not compromise the toxicity of the toxin. Thus the aforementioned ellipticine conjugate was very active in spite of the incomplete removal of linker amino acid residues, but a similar conjugate of the peptoid toxin, hemiasterlin, was totally inactive because that natural product requires an unmodified C-terminus. (M.Dyba, unpublished data).

The number of small molecule toxins that can be used for the construction of substrates for receptor-mediated delivery to cancer cells is not large when one considers all of the criteria outlined above.

Alkylating agents

Compounds such as nitrogen mustards, nitrosoureas, triazenes can be readily attached to receptor ligands and a number of such constructs have been described [138, 229, 230, 226]. These compounds, in general, are not sufficiently potent to be effective as drugs. Typically, their toxicities are in the micromolar range, which, given the low density of receptors on the tumor cell surface, is not high enough to produce a desired anti-tumor effect at acceptable drug concentrations. However, nitrogen mustard conjugates have been used successfully in combination with ligands directed to nuclear receptors [231].

Synthetic Toxins

A number of highly toxic synthetic molecules have been described that could be useful when conjugated with peptide ligands. The ellipticine analog 1 (Fig. **7**), a synthetic derivative of the natural product has already been mentioned as an almost ideal drug for conjugation. It is highly toxic (IC_{50} in picomolar to low nanomolar range, depending on the tumor). It is very chemically and metabolically stable and is readily conjugated with ligands [173, 174].

The synthetic analogs of the anti-tumor antibiotics CC1065 and duocarmycin offer some interesting possibilities for conjugation. The synthetic duocarmycin derivative DU-257 (Fig. **7**) [232] was linked to a monoclonal antibody through the convenient aminoethyl side chain. Similar chemistry could be used for the construction of a peptide

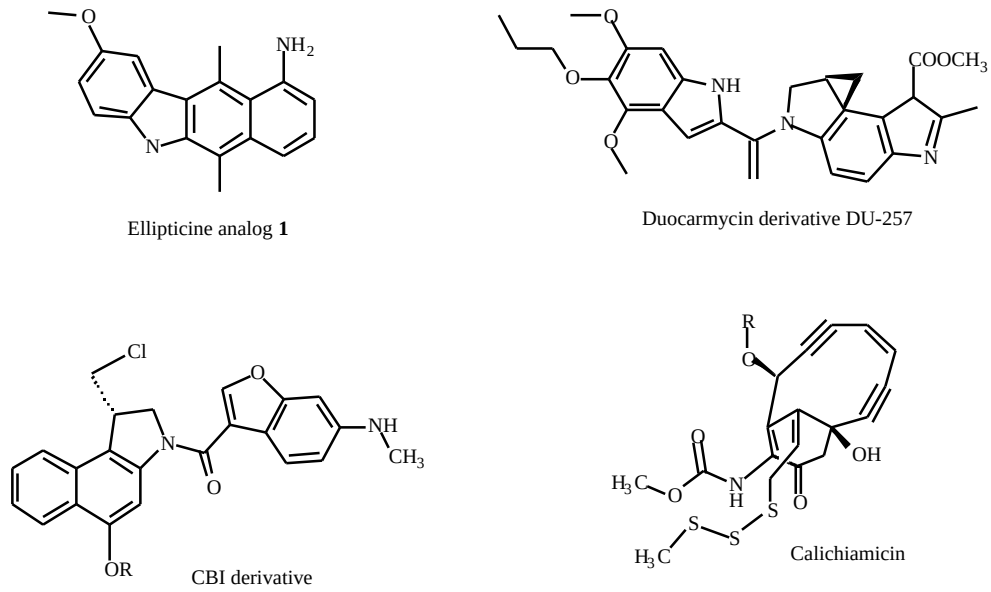


Fig. (7). DNA-binding compounds suitable for generation of receptor-targeted toxins.

conjugate. Interestingly, the enzymatic processing of the peptide derivative of DU-257 left a valine residue on the molecule, which did not compromise its activity. Synthetic derivatives of CC1065, such as the CBI (Fig. **7**) are also very toxic [233]. Additionally, CBI is an amino acid and can be readily linked to peptides. These compounds are not readily available and their syntheses are not trivial. However they have the required chemical properties to be effective receptor-targeted toxins.

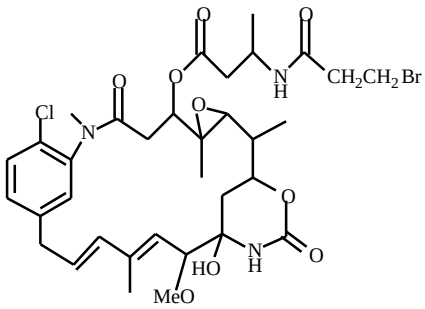
Toxins Derived from Natural Sources

Nature has provided an abundance of highly toxic materials that have been used in cancer chemotherapy. The effectiveness of many of these compounds is compromised by severe toxic side effects. Here we list a few of these compounds that are good candidates for linking to receptor ligands.

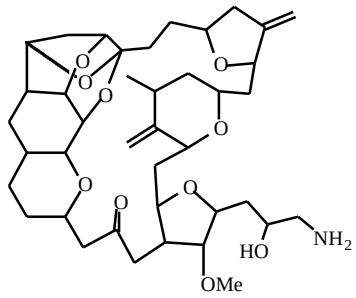
Anthracyclins, such as doxorubicin and daunomycin (Fig. **1A** and **B**) have been linked to numerous carriers [28, 35, 39, 120, 131-135, 172, 234]. As was mentioned above, we prepared several doxorubicin derivatives of heptagastrin. In one case, the ligand was attached via the glucosamine amino group, using a carbamate link. This compound was inactive, probably because the lysosomal proteases failed to remove the substitution on the glucosamine nitrogen, although the compound was taken up by the cells via endocytosis. This nitrogen must be unsubstituted for optimal activity [235]. In another case, we derivatized the doxorubicin at C-14 (i.e., 9-hydroxyacetyl group) to heptagastrin through a thiol linker. This compound was taken up into receptor-negative cells at about the same rate as into receptor-positive cells. Such passive internalization is not an isolated problem [236] and is not accounted for by simple hydrophobicity/ hydrophilicity criteria, as suggested earlier [239]. Nevertheless, a number of successful links of anthracylines to peptide hormones are known [28, 131, 132]. The great advantage of linking anthracyclines to peptide carriers is that receptor mediated delivery obviates the problems of multiple drug resistance and the severe side effects such as cardiotoxicity.

Another group of compounds that are especially attractive in this regard are the natural product antibiotics, the enediynes, which include calicheamycins, esperamycins, and neocarzinostatins [237, 238]. The enediynes exert their picomolar cytotoxic effects through highly specific DNA damage that is produced by a very unusual diradical, formed during a thiol-catalyzed rearrangement of the enediyne core. The enediynes have generated considerable research activity because of their unique mechanism of action, but their extremely high toxicity remains a problem for clinical applications. It would be very useful therefore to prepare conjugates of an enediyne that would be recognized by a receptor. Simple conjugation will not be sufficient in this case because there are many substances in general circulation (e.g., GSH) that will activate the compound, conjugated or not, before it reaches a target cell. A MoAb conjugate of calicheamycin was made through the trisulfide functionality, the site for the reaction with thiol, which initiates the activation cascade [148-157]. This is a very interesting approach, because it prevents the premature activation of the drug by decreasing the electrophilicity of that site. Clearly, this research will require considerable developmental chemistry before a successful linker can be produced, but there are some good models to follow.

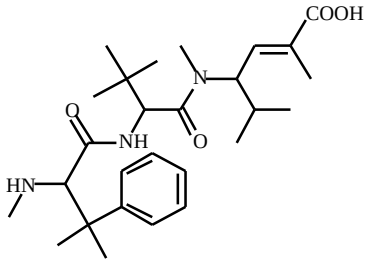
Paclitaxel and its derivatives (Fig. **2A**) [239] represent a series of potent mitosis inhibitors that exert their action by stabilizing microtubules. Several of these compounds have been linked to targeted delivery molecules, including MoAbs [217, 220, 240].



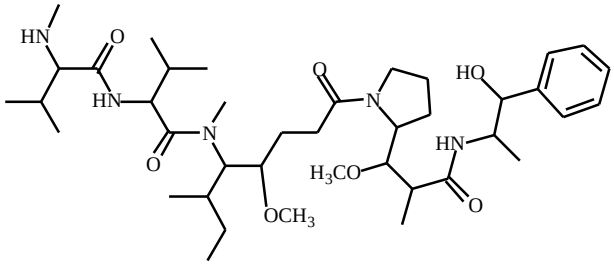
Maytansinoid DM1



Halichondrin derivative E7389



Hemiasterlin derivative HTI286



Monomethylauristatin E (MMAE)

Fig. (8). Tubulin-binding toxins.

Camptothecins (Fig. **3C**) [241] are very active anti-tumor agents that specifically inhibit topoisomerase I by stabilizing the so-called cleavage complex [242]. Since topoisomerase I is involved in many cellular processes, the camptothecins exhibit severe toxic side effects. However, some of these compounds can be attached to peptide hormones for selective delivery to cancer cell [210].

Maytansinoids, derivatives of microbial metabolites isolated from a variety of plants are extraordinary potent tubulin-binding agents [243]. Maytansinoid DM1 (Fig. **8**) developed by Immunogen was used for conjugation to a number of cancer targeting monoclonal antibodies through a disulfide linker [68]. The resulting conjugates have produced very impressive results in the animals and showed therapeutic effect in phase I clinical trials [69].

Several other tubulin binding compounds derived from naturally occurring toxins have sufficient potency and structural properties that allow their application as warheads in receptor-targeted anti-cancer drugs. Simplified analog of halichondrin B, E7389 (NSC #707389, Fig. **8**) developed by Eisai Corporation has a free amino group that can be derivatised without effecting the activity [244] and thus attached to a peptide linker and a ligand.

Dolastatins and hemiasterlins, peptoid tubulin binding toxins originally isolated from marine organisms [245] also present tempting opportunities for the use in targeting to tumor-specific receptors. Dolastatin 10 analog monomethylauristatin E (MMAE, Fig. **8**) was recently coupled to Lewis Y antigen–specific monoclonal antibodies in an elegant approach utilizing methylated N-terminus of the toxin and a self-eliminating cathepsin B-sensitive linker [246]. The conjugate induced remarkable regressions of established tumor xenografts in mice with therapeutic indices as high as 60-fold. Evidently, similar mode of attachment can be used for MMAE coupling to other cancer-specific ligands.

ABBREVIATIONS

(2-17) (15-Nle) HG	=	Human gastrin I fragment 2-17 with Nle in 15-position
(5-Nle) 7G	=	Heptagastrin peptide with Nle in fifth position
[C^{15}]NPY	=	15-amino acid fragment from C-terminus of neuropeptide Y
[C^{8}]-hCT(9-31)	=	Oktopeptide fragment 9-13 from human calcitonin
5-FU	=	5-fluorouracil
ADM	=	Adriamycin
BBN	=	Bombesin
BBN[7-13]	=	Bombesin fragment 7-13
BR96	=	BR96 monoclonal antibody
BrdU	=	Bromodeoxyuridine
C225	=	C225 monoclonal antibody
cAC10	=	cAC10 chimeric monoclonal antibody

CALLA	=	Common acute lymphoblastic leukemia antigen
Chg	=	L-α-cylohexylglycine
Cit	=	Citrulline
CPT	=	Camptothecin
DCC	=	Dicyclohexylcarbodiimide
DMF	=	*N*,*N*-dimethylformamid
DMHBA	=	3,5-dimetyl-4-hydroxybenzyl alcohol
DOX	=	Doxorubicin
DTT	=	Dithiothreitol
Ell	=	1-[3-[N-(3-aminopropyl)-N-methylamino]propyl]amino-9-methoxy-5,11-dimethyl-6H-pyrido[4,3-b]carbazole
EPR	=	Enhanced permeability and retention effect
GEM	=	Gemcitabine
Gly-α(5-FU)	=	2-(5-fluorouracil-1-yl)-L-glycine
HPMA	=	*N*-(2-hydroxypropyl)methacrylamide-based copolymers
Hyp	=	L-*trans*-4-hydroxyproline
IGR-39	=	IGR-39 human melanoma cell line
Karpas 299	=	CD30+ anaplastic large cell lymphoma cell line
L2987	=	L2987 human lung adenocarcinoma cell line
MAb	=	Monoclonal antibody
MCF-7	=	MCF-7 breast cancer cell line
MDA-MB-435	=	MDA-MB-435 human breast carcinoma cell line
MMAE	=	Monomethyl auristatin E
MMP2	=	Matrix metalloproteinases 2 (gelatinase A)
MMP9	=	Matrix metalloproteinases 9 (gelatinase B)
MTEG	=	Methoxytriethylene glycol
MTX	=	Methotrexate
Mu	=	Morpholinocarbonyl
NPY	=	Neuropeptide Y
P67.6	=	P67.6 monoclonal antibody
PABC	=	*p*-aminobenzylcarbonyl
PABE	=	*p*-aminobenzyl ethers
PEG	=	Polyethylene glycol
PHEA	=	α,β-poly(*N*-hydroxyethyl)-DL-aspartamide polymer
PHPMA	=	Random copolymers of N-(2-hydroxypropyl)methacrylamide
PK2	=	Conjugate of *N*-(2-hydroxypropyl)methacrylamide copolymer with doxorubicin-galactosamine
PSA	=	Human prostate specific antigen enzyme
PXT	=	Paclitaxel
RGD-4C	=	CDCRGDCFC peptide
Suc	=	Succinic acid
SU-DHL-1	=	SU-DHL-1 human lymphoma anaplastic large cell line
VIP	=	Vasoactive intestinal peptide
WGA	=	Wheat germ agglutinin
Z	=	Benzyloxycarbonyl

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