

Peptide-Mediated Targeted Drug Delivery

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Abstract: Targeted drug delivery to specific group of cells offers an attractive strategy to minimize the undesirable side effects and achieve the therapeutic effect with a lower dose. Both linear and cyclic peptides have been explored as trafficking moiety due to ease of synthesis, structural simplicity, and low probability of undesirable immunogenicity. Peptides derived from sequence of cell surface proteins, such as intercellular adhesion molecule-1 (ICAM-1), LHRH, Bombesin, and LFA-1, have shown potent binding affinity to the target cell surface receptors. Moreover, peptides derived from ICAM-1 receptor can be internalized by the leukemic T-cells along with the conjugated moiety offering the promise to selectively treat cancers and autoimmune diseases. Systematic analyses have revealed that physicochemical properties of the drug–peptide conjugates and their mechanism of receptor-mediated cellular internalization are important controlling factors for developing a successful targeting system. This review is focused on understanding the factors involved in the development of an effective drug–peptide conjugate with an emphasis on the chemistry and biology of the conjugates. Reported results on several promising drug–peptide conjugates have been critically evaluated. The approaches and results presented here will serve as a guide to systematically approach targeted delivery of cytotoxic drug molecules using peptides for treatment of several diseases. © 2010 Wiley Periodicals, Inc. *Med Res Rev*, 32, No. 3, 637–658, 2012

Key words: peptide; targeted delivery; receptor; endocytosis; conjugation; ICAM-1; LFA-1; bombesin

1. INTRODUCTION

Targeted drug delivery methods have been explored to improve drug efficacy and lower side effects by directing the drug to a specific cell type. To date, many strategies have been investigated to accomplish this goal, and some of these strategies rely on the differences between the cellular compositions of the targeted cells and those of the nontargeted cells. These differences could be in the expressed surface receptors (i.e., the absence or presence of certain receptors), the metabolism profiles (i.e., different enzyme expression or intracellular trafficking), the site/location of the cells (i.e., circulating in blood stream vs. organ), and the

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nature of the cells (i.e., normal vs. cancerous). For example, tumor cells have certain up-regulated receptors, enzymes, and other metabolic features that are not present in normal cells in the body. These differences can be used to discriminate the delivery of a drug to diseased cells rather than to normal cells. Furthermore, the differences in cellular trafficking profiles and the pH of endosomes between normal and cancer cells have also been exploited for selective drug delivery to a specific compartment in the intracellular space of cancer vs. normal cells.¹

Peptides (e.g., Arg-Gly-Asp (RGD) peptides,² poly-Arg peptides),^{3,4} proteins (e.g., antibodies,⁵ transport proteins, and transferrin⁶), and small molecules (e.g., folate⁷) have been used to selectively direct drugs to cancer cells with up-regulated receptors by forming drug-carrier conjugates (Fig. 1). However, none of the drug-peptide conjugates has successfully reached the market; this may be due to various factors, including (a) the difficulty in developing the appropriate ligand for the targeted receptor(s) on the cell surface, (b) the lack of understanding of the receptor recycling and trafficking mechanisms, (c) insufficient information on the mechanisms of uptake and disposition of the ligand inside the cells, (d) a lack of understanding of the pharmacokinetic and pharmacodynamic profiles of the drug-peptide conjugate, and (e) the limited number of systematic studies on the relationships between the physicochemical and transport properties of the conjugates. Thus, there is a need to study these aspects of the drug-peptide conjugates to increase the probability of success of these molecules in clinical settings. Ideally, conjugation of the drug to the targeting peptide should not interfere with the recognition of the peptide by its receptor(s). In addition, the

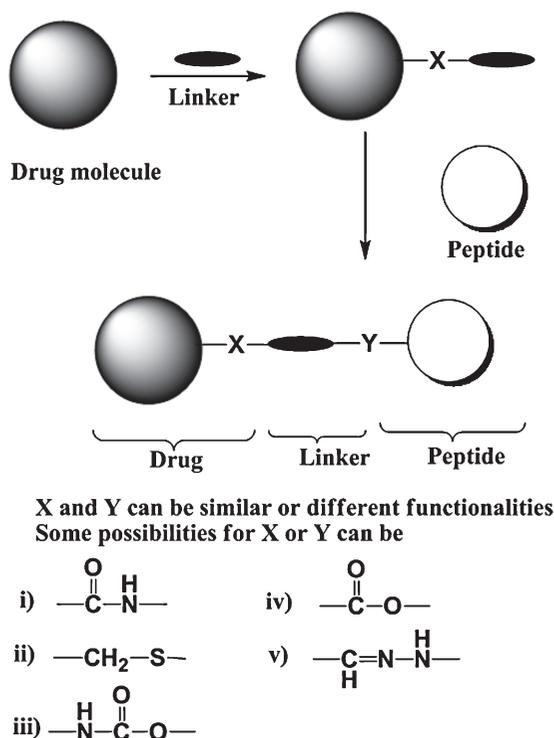


Figure 1. The structure of a drug-linker-peptide conjugate. X and Y represent the common functional groups used to connect either the drug or the peptide to the linker. X may be similar to or different than Y. Here, the primary focus is on the nature of the X bond, and the drug peptide conjugation chemistry has been classified according to the nature of the X bond: (i) amide, (ii) thioether, (iii) carbamate ester, (iv) carboxylic acid ester, and (v) hydrazone bond.

pharmacologic or cytotoxic property of the drug should be maintained when the drug is conjugated to the carrier molecules.

This review illustrates the chemistry, biology, and utilization of small drug–peptide conjugates for selectively delivering the drugs to a specific group of cells (Fig. 1). The first section discusses the attributes of an ideal peptide carrier and the drug. Emphasis will be placed on cyclic peptides and their possible conjugation sites. The second section explores the chemistry behind the drug–peptide conjugation via different types of chemical bonds, including carboxylic acid ester, amide, carbamate ester, hydrazone, and enzymatically cleavable bonds. The presence of a spacer between the drug and the peptide carrier may be necessary to ensure recognition of the carrier by the receptor. The third section focuses on the families of peptides that have been conjugated to drugs and their evaluation in *in vitro* and *in vivo* biological systems.

Drug–peptide conjugates are most often administered via the parenteral route because they may not be efficiently delivered orally. The physicochemical properties of peptides (i.e., size, hydrophilicity, and hydrogen-bonding potential) make it difficult for them to cross the intestinal mucosal barriers. In addition, proteases on the surface of intestinal mucosa brush border membranes rapidly metabolize the peptide carrier into fragments. In parenteral delivery, the conjugates travel through the systemic circulation and are distributed to the small capillaries that carry them to the target cells. Upon peptide–receptor interaction, the conjugate can potentially undergo receptor-mediated endocytosis, in which the conjugate may move through the early and late endosomes and finally into the lysosome (Fig. 2). The receptors are either separated from the conjugates in the early sorting endosomes or move to the lysosomes along with the conjugate. After separation from the conjugates, the receptors may be recycled onto the cell surface for the next round of transport. There is a significant drop in pH in the lysosome that may affect the binding between the conjugate and the receptor. It can also affect the linkage between the drug and the peptide (Fig. 2). As an example, the transferrin receptors bind and carry the transferrin protein to transport iron into the cells and, after pH change in the endosomes, the transferrin releases iron and the receptor is recycled back to the cell surface.⁸ The mechanisms of receptor uptake have been shown to occur via clathrin coat-⁹ or caveolin-mediated endocytosis (i.e., CD11a/CD18).¹⁰

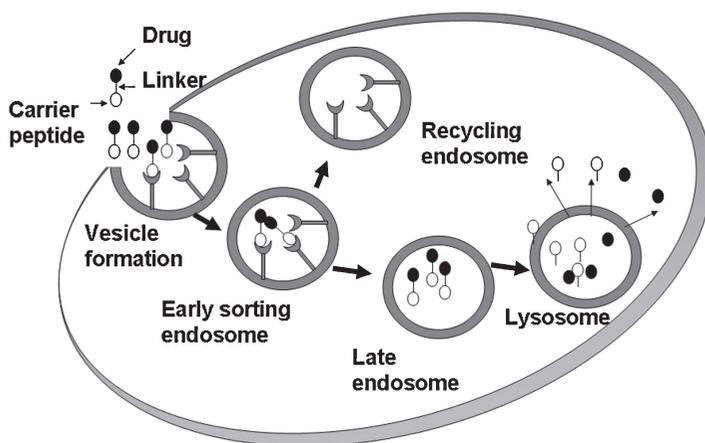


Figure 2. Hypothetical schematic of one possible cellular-internalization process for a drug–peptide conjugate. Drug–linker–peptide interacts with the cell surface receptor for internalization via receptor-mediated endocytosis. Early sorting endosomes may separate the receptor from the conjugate and recycle receptors to cell surface. The conjugate moves to cellular compartments with progressively lower acidity leading to separation of the drug from the carrier. The drug is then released into the cytoplasm to reach the target site for the drug.

2. DRUG-PEPTIDE CONJUGATES

A. Peptide Carrier

There are several important requirements for using peptides as carrier molecules. First, the peptide should selectively bind to the cell surface receptors on the target cells. Second, the receptor should be expressed only on the target cells or the expression should be higher in the target cells than in the nontargeted cells. Third, the peptide carrier should be sufficiently stable in the systemic circulation to reach the target cells at an effective concentration. Fourth, selection of the site of conjugation on the peptide is critical for retaining its binding properties to the receptor, because drug conjugation may impose a steric hindrance that interferes with receptor recognition.

Peptides as carriers may offer some advantages over proteins because peptides are relatively easy to modify for generating derivatives and to produce in large quantities. The drug-peptide conjugate is easier to analyze than a drug-protein conjugate. Due to the absence of tertiary structure in peptides, they often do not undergo physical degradation as do proteins. In addition, the number of drug molecules and their conjugation regioselectivity can be controlled; in contrast, it is difficult to control the number and regioselectivity of the conjugated drugs on a protein. Because the drugs are often conjugated to the side chains of reactive residues (e.g., Lys or Asp), the conjugates consist of a distribution of products with various numbers of drugs attached to various sites of individual protein molecules. Thus, the formation of the conjugates may be difficult to reproduce, and the resulting products can also be difficult to analyze. In addition, peptide-drug conjugates may have lower immunogenicity than protein-drug conjugates.¹¹ The immunogenicity of protein-drug conjugates may arise from the presence of nonnative protein conformers or protein aggregates upon drug conjugation.

In many cases, cyclic peptides have higher selectivity for the receptor than do the parent linear peptides because cyclic peptides have more restricted conformations. However, it is still possible that the formation of the cyclic peptide may force the adoption of an unfavorable conformation for binding to the receptor. Cyclic peptides are prepared by three different cyclization methods: (1) backbone-to-backbone, (2) backbone-to-side chain, and (3) side chain-to-side chain (Fig. 3). The backbone-to-backbone cyclization is formed using an amide bond between the *N*- and *C*-termini (Conjugates 1 and 2). In this case, the side chain of Lys, Asp, or Glu is utilized as the conjugation point between the drug and the peptide (Conjugates 1 and 2). It is preferable that the amino acid for conjugation site should not be part of the receptor recognition sequence. The backbone-to-side chain cyclization is formed by connecting the *N*-terminus with the carboxylic acid side chain of Asp or Glu residue by an amide bond, and the drug is conjugated to the *C*-terminus of the peptide (Conjugate 3). Alternatively, the peptide *C*-terminus is linked to the side chain of the Lys residue, while the drug is conjugated to the *N*-terminus of the peptide (Conjugate 4). Finally, the side chain-to-side chain cyclization can be formed by using an amide bond or a disulfide bond. A peptide bond between the Asp side chain and the amino group of a Lys residue produces a cyclic peptide with an open *C*- or *N*-terminus that can be utilized to conjugate the drug (Conjugates 5 and 6). A cyclic peptide is also made by linking the side chains of two Cys residues to make a disulfide bond and the drug is conjugated to either the *N*- (Conjugate 7) or *C*-terminus (Conjugate 8). A Cys residue can be inserted within the sequence of a cyclic peptide, and the thiol group of the Cys residue can be utilized for linking the drug to the peptide (Conjugate 9).

B. Examples of Drug Molecules

The drug molecules described here are classified as cytotoxic small molecules, such as doxorubicin (DOX), methotrexate (MTX), and camptothecin (CPT). These molecules have side

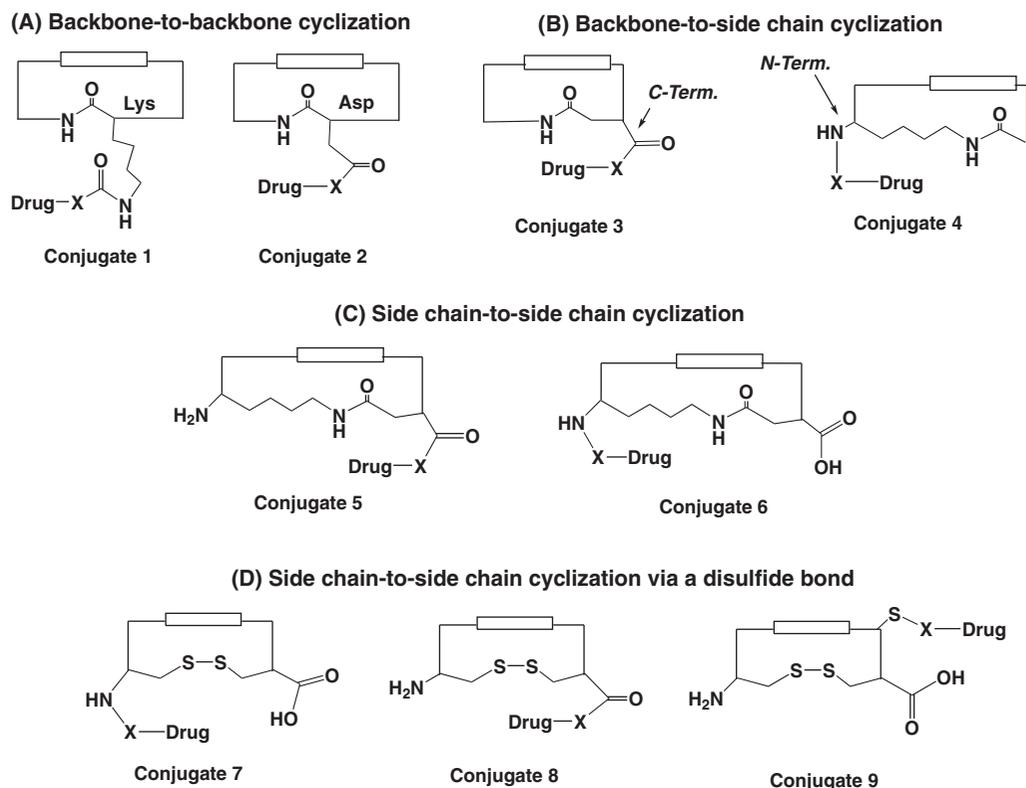


Figure 3. (A) Conjugates **1** and **2**: Cyclic peptides formed by backbone-to-backbone cyclization with side chains of Lys and Asp available for drug conjugation. (B) Conjugates **3** and **4**: Cyclic peptides formed by backbone-to-side chain cyclization. *N*- and *C*-termini of the peptide can be conjugated to the Asp and Lys side chains, respectively. Free *C*- and *N*-termini can then be conjugated to the drug. (C) Conjugates **5** and **6**: Cyclic peptides formed by side chain-to-side chain cyclization via amide bond. Side chains of Lys and Asp can be conjugated, and the free *C*- and *N*-termini can be used for drug conjugation. (D) Conjugates **7**, **8**, and **9**: Cyclic peptides formed by side chain-to-side chain cyclization. Cysteine side chains can be conjugated via disulfide bond. Free *N*-terminal, *C*-terminal, and free Cys present in the sequence can be used for drug conjugation.

effects because they are taken up by both cancerous and normal cells. Due to the physico-chemical properties of DOX and CPT, these drugs enter the cells via a passive diffusion mechanism by readily partitioning into the cell membranes. On the other hand, MTX enters the cell using uptake transporters, such as reduced folate carrier and membrane folate binding protein (MFBP).¹² The selectivity of MTX for cancer cells over normal cells occurs because cancer cells have upregulated expression of MFBP. Nonetheless, MTX can still be internalized by normal cells to produce side effects. Thus, conjugation of any of these drugs (i.e., DOX, CPT, or MTX) to a peptide carrier may direct them to a specific population of cells and lower the drug side effects. As in peptides, the site of conjugation on the drug molecule is an important consideration for maintaining the drug activity. If the conjugation has to be done at the active functional group, a cleavable prodrug moiety may be used to link the drug to the peptide so that the free drug can be released from the carrier. One potential drawback of using a labile linker is that the drug may be released prematurely before reaching the target cells.

1. Doxorubicin (DOX)

DOX is widely used as a model drug for conjugation to different carriers, such as peptides,^{13,14} antibodies,^{15,16} polymers,^{17,18} proteins,^{19,20} and polymer beads.²¹ The primary mechanism of action of DOX is DNA-intercalation and inhibition of topoisomerase II

during DNA synthesis.²² DOX can also exert cell toxicity through the generation of hydrogen peroxide, which damages the membranes of mitochondria.²³ For its activity as a DNA intercalator, it is necessary for the DOX molecule to enter the nucleus; ideally, the targeting system should carry the drug into the nucleus. However, conjugation of DOX to the carrier peptide may prevent DOX from entering the nucleus.²⁴ To overcome this problem, DOX has been conjugated to the carrier through a cleavable linker, which allows the drug to be released from the conjugate.

Several functional groups on the DOX molecule have been used for conjugation to the carrier molecules. The primary amine of the sugar moiety can be directly linked to a carboxylic acid group of the C-terminal or the Asp side chain on the peptide carrier. To provide a spacer between the DOX and the peptide carrier, the amino group is reacted with succinic or glutaric anhydride to give a product with a free carboxylic acid group, which can be linked to a free amino group on the peptide. The primary alcohol at the C14 of DOX has been linked to a carboxylic acid group on the carrier molecule via an ester bond. The C13 ketone group on DOX is also reacted to hydrazine to form a hydrazone spacer that is linked to the peptide carrier.

2. Methotrexate (MTX)

Many studies have been done to conjugate MTX to different carriers for improving its delivery and efficacy. This drug is used for the treatment of rheumatoid arthritis and several different carcinomas, including acute lymphocytic leukemia, lymphomas, and choriocarcinoma. The effectiveness of this drug depends on the efficiency of the active transporters on the target cell. One of the mechanisms of action of MTX is inhibition of the activity of dihydrofolate reductase (DHFR) enzyme that subsequently blocks thymidine synthase for DNA synthesis. In addition, the MTX is trapped inside the cell by forming MTX polyglutamate adducts. In many cases of cancer therapy, MTX therapy is ineffective at low doses; at high doses, this drug is effective but generates side effects due to its cytotoxicity to normal cells.

Targeting of MTX using antibodies and peptides has been explored to increase its effectiveness.^{25,26} The MTX molecule is conjugated to the carrier molecule through the γ -carboxylic acid group of the glutamic acid residue to maintain its activity.²⁷ The α -carboxylic acid of MTX is necessary for binding to DHFR; thus, conjugation via the α -carboxylic acid is not desirable. To conjugate MTX to the carrier molecule, the α -carboxylic acid of the Glu residue is protected to give MTX- α -OtBu. After coupling of the γ -carboxylic acid with the N-terminal of the peptide carrier, the tertiary-butyl protecting group can be removed to give the desired conjugate.²⁷

3. CHEMISTRY OF DRUG-PEPTIDE CONJUGATION

Different approaches have been used to conjugate carrier peptides to cytotoxic drugs. The chemistry of conjugation has a profound impact on the stability of the conjugate. Each functional group to link the peptide and the drug has both advantages and disadvantages. Thus, this section explores common conjugation methods that have been used to make drug-peptide conjugates. The chemical bonds between the drug and the spacer will be discussed. The same chemical bonds are normally used to link the spacer and the peptide carrier or to link the drug directly to the peptide without a spacer.

A. Amide Bond

Drug-peptide conjugation via an amide bond is carried out by linking the carboxylic acid of the drug and primary amine of the spacer/peptide. The chemistry of the amide bond

formation is straightforward, and the bond has relatively high chemical stability. Because the enzymatic cleavage of the amide bond between the drug and the peptide may be slow, there is a high probability that the conjugate will reach the target site with minor degradation. In this case, the drug portion of the conjugate may be active while it is attached to the peptide carrier. If the drug is attached through a functional group that is necessary for its activity, a cleavable promoiety can be incorporated between the drug and the peptide, by which the drug can be released from the carrier peptide by pH change or enzymatic reaction (i.e., esterase). For amide bond formation, the carboxylic acid on the spacer, peptide, or the drug can be activated with *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluorophosphate (HBTU) or a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxybenzotriazole (HOBT). The activated carboxylic acid is then reacted with an amine group of the counterpart (i.e., drug or peptide) in the presence of a strong base (i.e., diisopropylethylamine or triethylamine) in different solvents, including dimethylformamide or dimethylsulfoxide to give the desired conjugate.

DOX has been conjugated to different peptides, including cIBR [cyclo(1,12)-PenPRGGSVLVTGC],²⁸ cIBR7 [cyclo(1,8)CPRGG SVC],²⁹ human calcitonin (hCT)-derived peptides,³⁰ and Vectocell peptides using an amide bond.³¹ For conjugation to cIBR peptide, the DOX was modified to DOX-hemisuccinate by reacting the amino group of DOX with succinic anhydride to generate free carboxylic acid (Fig. 4; pathway a). The free carboxylic acid of DOX-hemisuccinate was activated and reacted with the *N*-terminus of cIBR peptide to produce DOX-cIBR conjugate. The *in vitro* stability of DOX-cIBR in human promyelocytic HL-60 cells showed that DOX-cIBR conjugate was stable; only 15% degradation was observed over a 24 hr period, indicating the high stability of the amide conjugation method.²⁸ DOX was also conjugated to human calcitonin (hCT(9–32); CLGTYTQDFNKFHTFPQ-TAIGVGAP-NH₂) through a maleimido-benzoic acid spacer. The amino group of DOX

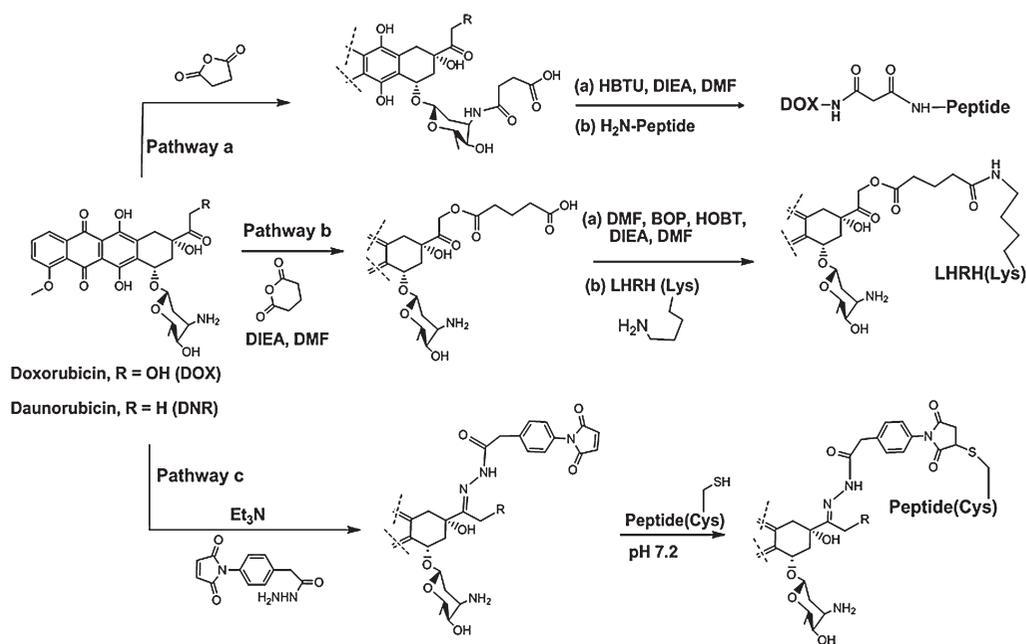


Figure 4. Different conjugation strategies are used to attach drug to peptide via linker. DOX and DNR are selected here as the drugs of choice. Pathway (a) shows the amide bond formation approach between the drug and the linker-peptide (adapted from²⁸). Pathway (b) depicts the carboxylic acid ester bond formation strategy between the drug and the linker-peptide (adapted from³³). Pathway (c) shows the hydrazone bond formation approach between the drug and the linker-peptide (adapted from³⁶).

formed an amide bond with the carboxyl group of the maleimido-benzoic acid spacer. Then, nucleophilic attack of the double bond of the maleimide group by the thiol of the Cys1 residue on hCT(9–32) peptide produced the desired conjugate.³⁰ A similar approach was used for conjugation of DOX to Vectocell peptides (i.e., CVKRGKLRHVRPRVTRMDV and SRRARRSPRHLGSGC) by amide bond formation with the carboxylic acid group of maleimidobutyric acid.³¹ Amide linkage was also used for conjugation of a β hairpin peptide (RWQYVpGKFTVQ) with an $\alpha_4\beta_1$ integrin recognition peptide sequence LDV at the *N*-terminus and 7-amino-4-carbamoylmethylcoumarin (a fluorescent probe for enzymatic hydrolysis) at the *C*-terminus. Enhanced rate of model-drug release was observed in the presence of live cells compared to that in the absence of cells. Possible conformational change due to receptor binding was proposed to be a factor for the release of drug in the presence of cells.³²

B. Carboxylic Acid Ester Bond

The ester linkage is commonly used to conjugate drug to peptide because it can be hydrolyzed chemically or enzymatically (i.e., esterase) to release the drug. However, due to the instability of the ester bond, the drug may be released before reaching the target tissues. Different analogs of luteinizing hormone-releasing hormone (LHRH) peptide were conjugated via an ester bond to various cytotoxic agents, including DOX and its derivatives. To conjugate DOX to LHRH peptide (Glp-HWSYkLRPG-NH₂), C14 of DOX was modified with glutaric ester and the other carboxylic acid of the glutarate was linked to the side chain amino group of D-Lys on LHRH peptide to give DOX-LHRH conjugate (Fig. 4; pathway b).^{33,34} The DOX-LHRH was quite stable in biological media and retained the cytotoxic property of DOX while maintaining its binding affinity to pituitary LHRH receptors.^{35,36} The C14 position of DOX was also conjugated via a glutaric acid spacer to the cyclic peptide CNGRC, using an ester bond to target the aminopeptidase N/CD13 receptor on the cell surface. In this case, the stability of DOX-CNGRC conjugate was dependent on the incubation matrix, which suggests that the DOX might be released before reaching the target tissue.³⁷ Thus, it is necessary to evaluate the stability of the ester drug–peptide conjugate in different biological matrices to ensure that the conjugate will reach the target tissue before releasing the drug component.

C. Hydrazone Bond

A hydrazone linkage can be utilized as an acid-labile bond for releasing the drug molecule from the conjugate upon a decrease in pH in tumor extracellular environments and in the lysosomes. Daunorubicin (DNR) and DOX with a ketone functional group at C-13 were derivatized with hydrazine maleimido spacers (i.e., *m*-maleimidobenzoic acid hydrazine or *p*-maleimidophenylacetic acid hydrazine) to give hydrazide intermediates (Fig. 4; pathway c). These maleimide intermediates were reacted with the thiol group of the Cys residue in neuropeptide Y (YPSKPDNPGEDAPACDLARYYSALRHYINLITRQRY-NH₂ or NPY) to give the respective conjugates.³⁸ The presence of an aromatic ring on the spacer provided the possibility to regulate the stability of the hydrazone bond.³⁸ This hydrazone linker released less than 10% of the free drug at pH 7.4 compared to 35–40% at pH 5.0 over a 24 hr period. In contrast, the amide bond linkage showed very little drug released at either pH value.³⁹ Using the hydrazide bond, DOX was also conjugated to thermally responsive elastin-like polypeptide in an effort to utilize the enhanced permeability and retention⁴⁰ effect; the optimum release kinetics were controlled by changing the length and hydrophobicity of the spacer.⁴¹

DOX has been conjugated by hydrazide linkage to the modified side chain of glutamic acids of a polyGlu-peg polymer (with 15 glu per conjugate molecule), resulting in the incorporation of 4 DOX molecules per targeting agent. Conjugation of an $\alpha_v\beta_6$ integrin targeting tetrameric peptide [H2009.1] at the other end of the polyGlu-peg molecule resulted in a conjugate that have binding affinity to the target receptor. However, it was only two-fold more cytotoxic to the integrin-expressing cells than cells without integrin. Comprehensive analyses indicated that a poor uptake of the conjugate into the cells was observed despite the high binding affinity of the conjugate to the target receptor and might be due to the peg-poly Glu-DOX portion of the molecule. Perinuclear localization of the internalized drug also showed possible poor drug release profile from the conjugate. This indicates that the structural properties of the molecules have important influence in final drug efficacy.⁴²

Interestingly, cytotoxicity of nontargeting DOX-HPMA copolymer was increased by 150-fold after conjugation of high affinity E-selectin binding peptide (Esbp) to DOX-HPMA in human immortalized vascular endothelial cells. DOX was conjugated to one end of HPMA using hydrazide linkage while cysteine-containing Esbp (CDITWDQLWDLMK) was conjugated to the other end of HPMA using amide linkage.⁴³

D. Enzymatically Cleavable Bond

For enzymatic release of the drug, a specific peptide sequence may be utilized as a cleavable spacer between the drug and the carrier. One of the most widely used spacer sequences is a specific peptide substrate for the prostate-specific antigen (PSA), a serine protease enzyme that is expressed at high levels by prostate tumors.⁴⁴ PSA is known to mediate hydrolysis of semenogelin-I with high specificity by cleaving the peptide bond between Gln349 and Ser350.^{45–48} After mutation studies, several peptide sequences were specifically cleaved by PSA at the Gln-Ser or Gln-Leu peptide bond; thus, two peptide spacers (Glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-OH⁴⁷ and Mu-His-Ser-Ser-Lys-Leu-Gln⁴⁹) were used to link between DOX and the carrier peptide (Fig. 5; pathways a and b). These conjugates were highly toxic due to the release of Leu-DOX and DOX (Fig. 5; pathways a and b).^{47,49} Leu-DOX had cytotoxicity against cancer cell lines with fewer side effects, such as cardiac toxicity.⁴⁹ It is noted that the direct conjugation of the amino group of DOX to Gln carboxylic acid (i.e., without the Ser or Lue residue at the C-terminal) failed to release free DOX by hydrolysis. It was also suggested that the presence of the carrier peptide might affect the specificity of the spacer for the proteolytic enzymes. Enzymatically cleavable sequences can drastically improve the activity of conjugates as demonstrated by the introduction of the matrix metalloproteinase (MMP) cleavable peptide sequence (PVGLIG with a cleavage site between G and L) between DOX and PEG. Without the cleavable peptide sequence, DOX-PEG conjugates did not release the drug (amide linkage chemistry) and did not show anti-proliferative activity against U87MG cells. However, the presence of the enzyme-labile linker made the conjugate to have cytotoxicity comparable to DOX itself.⁵⁰

In a separate account, MMP-activated peptide-DOX conjugates have been shown to preferentially release DOX in tumor relative to heart tissue when administered to mice with HT1080 xenografts. The conjugates also prevented tumor growth with less marrow toxicity than DOX.⁵¹

Peptide-based biomaterials provide another promising application of enzymatically driven targeted drug delivery. 6-u-8 peptide has been synthesized by covalently incorporating a peptide substrate (SGRSANA) for urokinase plasminogen activator (uPA) between two β -sheet forming sequences. β -sheet stacking via solvent exchange led to self-assembled matrix formation. A separate peptide B-r7-kla was synthesized by covalently incorporating a mitochondria disrupting peptide sequence (klaklakklaklak) in between a β -sheet sequence and

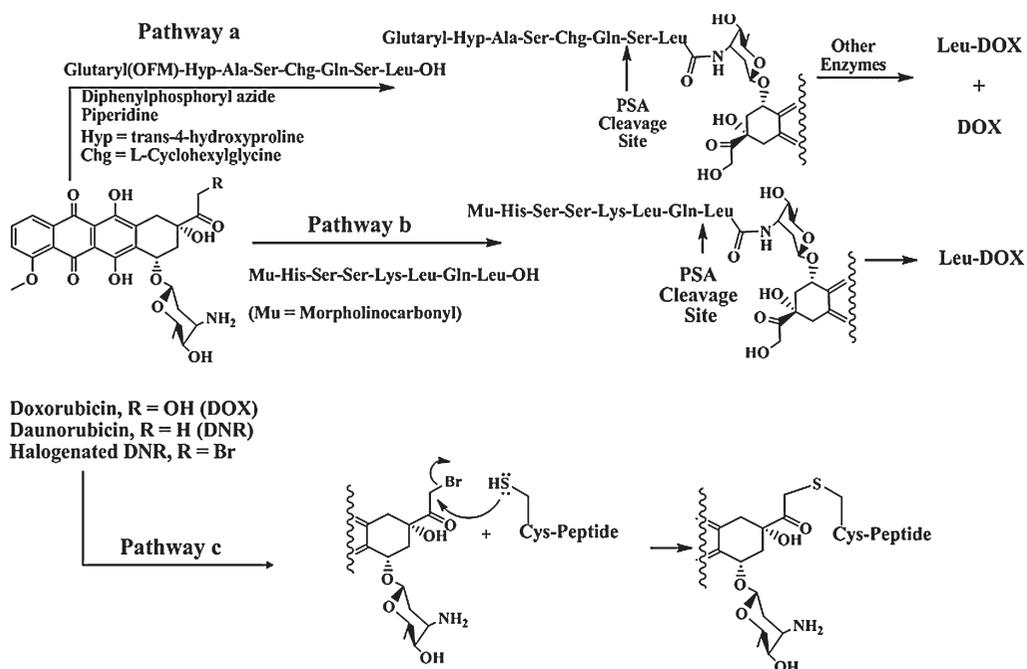


Figure 5. Different conjugation strategies are used to attach drug to peptide. DOX, DNR, and halogenated DNR are selected here as the drugs of choice. Pathways (a) and (b) show the enzymatically cleavable [prostate specific antigen (PSA) cleavable] bond formation approach between the drug and the carrier peptide (adapted from^{47,49}). Pathway (c) shows the thioether bond formation strategy between the drug and the carrier peptide (adapted from³¹).

a cell-penetrating sequence. Then, B-r-7-*kla* peptide was encapsulated in the matrix of 6-u-8 peptide to demonstrate the feasibility of drug delivery potential. Significant cytotoxicity against HT-1080 cells was observed only in the presence of high uPA concentration, indicating the possible release in the vicinity of tumors with high enzyme level.⁵²

E. Thioether Bond

The thioether bond is chemically stable, such as the amide bond, and as a result the release of free drug is impeded by this conjugation process. Peptides containing a free thiol group, such as Vectocell peptides (i.e., CVKRGKLRHVRPRVTRMDV and SRRARRSPRHLGSGC), have been conjugated with drugs using a thioether linkage because these peptides are translocated across the cell membranes in an energy-dependent manner. Vectocell peptides were conjugated to DNR through nucleophilic displacement of the halogen on DNR to make a thioether link (Fig. 5; pathway c). These conjugates have been found to be significantly less cytotoxic than the free drug.³¹ This may be due to several factors. First, the presence of the peptide carrier may prevent the recognition of DOX. Second, the DOX–Vectocell bond is very stable; therefore, DOX cannot be released from the carrier. It is also possible that the peptide cannot be effectively digested in the lysosomes to release all the delivered DOX. Third, although the DOX is structurally exposed for activity, the intact conjugate may not be able to enter the nucleus.

A thioether bond was used to conjugate oligodeoxynucleotides (ODNs) to peptide carriers to improve their cellular uptake. In this case, 5'-thiol-derivatized phosphorothioate-ODNs against the protooncogene *bcl-2* was conjugated to a cyclic somatostatin analog with a maleimide group at the *N*-terminus (maleimide-cyclo(2,7)-fCYwKTCT) to form the thioether conjugate.⁵³ A thioether linkage was used to conjugate hydroxymethylacylfulvene

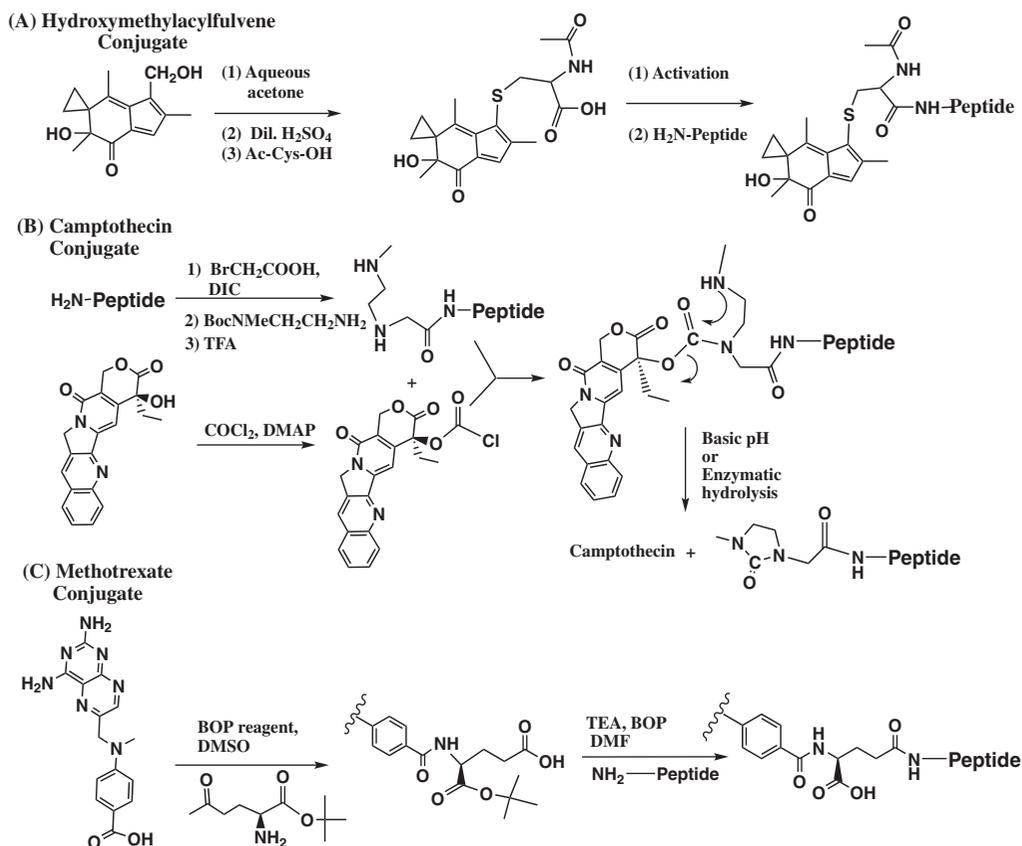


Figure 6. (A) Hydroxymethylacylfulvene is conjugated with Cys-containing peptides using thioether linking strategy (adapted from⁵⁴). (B) Camptothecin is conjugated with peptides using a carbamate ester bond to ensure release of the drug from the conjugate in a controlled manner (adapted from⁵⁶). (C) Methotrexate was conjugated with peptides using the α -carboxylic acid of the glutamic acid residue of the drug (adapted from^{25,34}).

(HMAF), an alkylating antitumor agent, to a cysteine-containing peptide (Fig. 6A). Through a thiol-displacement reaction, HMAF was first conjugated to N-acetylcysteine, and then the carboxylic acid of the N-acetylcysteine was activated and reacted with the *N*-terminus of the carrier peptide for targeting tumor cells.⁵⁴

F. Carbamate Ester Bond

A carbamate bond has higher stability in plasma than an ester bond, providing a higher probability of the conjugate reaching the target site.⁵⁵ CPT, and combretastatin (CBT) were conjugated to somatostatin analog peptides using a carbamate bond between the drug and the spacer (Fig. 6B for CPT).⁵⁶ The spacer contained a methyl-aminoethyl moiety that was attached to the carbamate nitrogen as a “built-in-nucleophile assisted releasing” (BINAR) moiety, which acted as a nucleophile to release CPT. This secondary amine of the BINAR moiety attacked the carbonyl carbon of the carbamate group to form a five-membered ring urea on the spacer; this was followed by the release of the drug into the medium. In this case, the pK_a value of the hydroxyl group of the drug influenced the rate of drug release from this conjugate. For example, CBT conjugate was highly unstable compared to CPT conjugate due to the pK_a of the alcohol in each drug. The CPT conjugate (Fig. 6B) was highly cytotoxic to the human neuroblastoma cell line IMR32 with a high expression of somatostatin receptor,

and the conjugate had half-lives of about 123 and 18 hr in phosphate buffer and rat serum, respectively. Modification of the length of the spacer influenced the stability of the conjugate. Although this approach has promise for selective delivery with adjustable rate of release, it can only be used for drugs with a hydroxyl group.^{56,57}

4. IN VITRO AND IN VIVO BIOLOGY OF DRUG-PEPTIDE CONJUGATES

A. Drug-Bombesin Analog Peptide Conjugates

Among the bombesin (BN) receptor subtypes, BN receptor Type 2 (also known as gastrin-releasing peptide receptor) may be the best choice for cancer cell-targeted delivery because it is upregulated in breast, prostate, small cell lung, and pancreatic cancers.⁵⁸ BN peptides have been conjugated to different drugs for targeting cancer cells. Receptor antagonist (RC-3095) and agonist (RC-3094) of BN receptors have been conjugated to DOX and 2-pyrrolino-DOX via a glutaric acid spacer (Fig. 7A, B). RC-3095 had higher binding affinity to the target receptor than RC-3094 on Swiss 3T3 cells; this is due to the presence of a hydrophobic D-Tpi residue on RC-3095 peptidomimetic. Conjugation of DOX or 2-pyrrolino-DOX to the less hydrophobic RC-3094 increased the conjugate binding affinity to the receptor about 1,000 times. In contrast, conjugation of DOX to the hydrophobic antagonist RC-3095 lowered its binding affinity to the receptor about two times. Nonetheless, DOX-RC-3095 conjugate preserved the DOX activity to inhibit cell growth of several human cancer cells, including

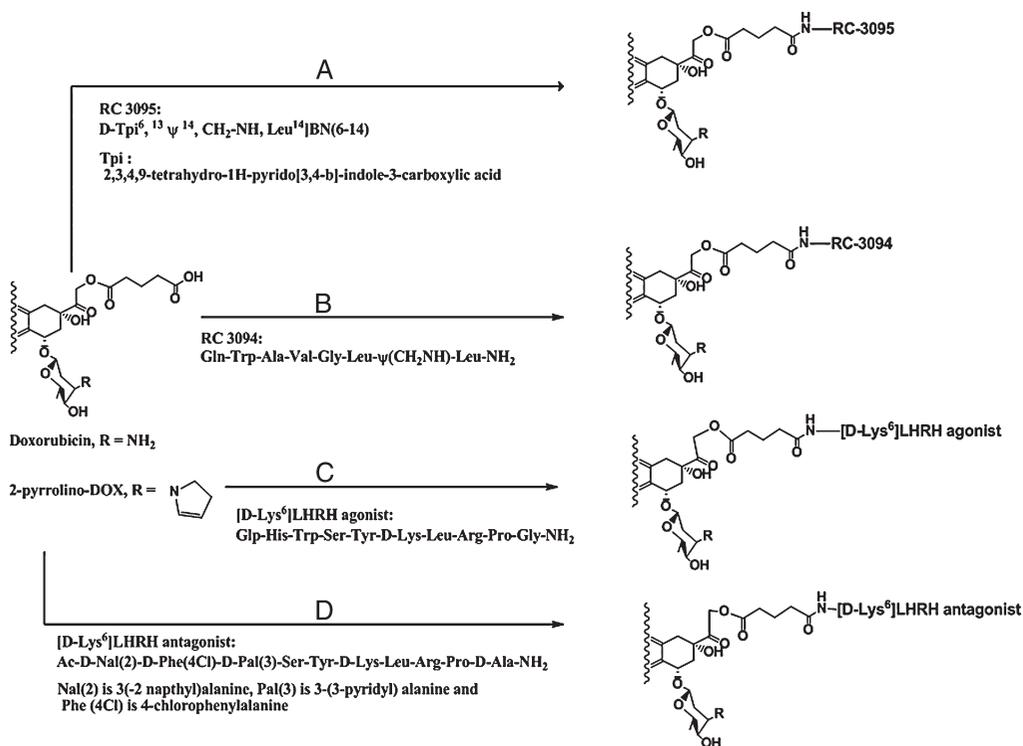


Figure 7. (A) and (B) show the conjugation of the *N*-termini of the bombesin antagonist peptide RC-3095 and RC-3094, respectively, to the C14 position of DOX and 2-pyrrolino-DOX using a glutaric acid spacer (adapted from⁵⁹). (C) and (D) show conjugation of D-Lys side chain of LHRH agonist and antagonist peptides, respectively, to the C14 position of DOX and 2-pyrrolino-DOX using a glutaric acid spacer (adapted from³³).

human pancreatic cancer (CFPAC-1), human SCLC (DMS-53), human prostate cancer (PC-3), and the human gastric cancer cell line (MKN-45).⁵⁹ The conjugate between RC-3094 and 2-pyrrolino-DOX showed significant antitumor activity with reduced toxicity in in vivo hamster nitrosamine-induced pancreatic cancer.⁵⁹ The conjugate activity could be inhibited by the parent peptide/peptidomimetic, suggesting that the conjugate is bound to BN receptor. However, the selectivity of the conjugate for cells that express BN receptor over the cells that do not express the receptor has not been evaluated. This study suggests that peptide–drug conjugation may influence the receptor-binding properties of the conjugate.

B. Drug–LHRH (GnRH) Analog Peptide Conjugates

Overexpression of LHRH receptors in breast, ovarian, endometrial, and prostate cancers provides an excellent opportunity to target drugs to these cancer cells using LHRH peptides.^{60,61} Short LHRH peptides have been designed as receptor agonists and antagonists for releasing luteinizing hormone.^{62,63} DOX and 2-pyrrolino-DOX have also been conjugated to LHRH agonist and antagonist peptides for selectively targeting cancer cells (Fig. 7C, D).³³ The presence of D-Lys at position 6 in LHRH peptide was necessary for high receptor binding affinity and agonistic activity (Fig. 7C); on the other hand, the hydrophobic tripeptide sequence at the *N*-terminal region of LHRH was necessary for the antagonist activity (Fig. 7D).^{62,63} Conjugation of DOX and 2-pyrrolino-DOX to the agonist maintained the binding affinity to the LHRH receptors. However, conjugation of the same drugs to the antagonist peptide lowered the binding affinity to the receptor about four-fold, suggesting that the drug moiety might interfere with the recognition of the peptide by the receptor. Nonetheless, the ability of LHRH-DOX conjugate to inhibit cancer cell growth (i.e., breast, prostate, mammary, and ovarian cancer cells) was comparable to that of the corresponding individual drugs.³³ In vivo studies indicated that the drug–agonist conjugates are less toxic and more potent than the corresponding individual drugs (i.e., DOX and 2-pyrrolino-DOX); this may be due to the selectivity of the conjugate to target cancer cells.

Polyethylene glycol (PEG; $M_w \sim 5,000$) has been utilized to link LHRH peptides and CPT to give CPT-PEG-LHRH for increasing conjugate solubility and retention in the systemic circulation.⁶⁴ CPT-PEG-LHRH conjugate was prepared by reacting the side chain of the Lys residue of LHRH peptide with *N*-hydroxysuccinimide-activated PEG-containing vinylsulfone (NHS-PEG-VS) (Fig. 8). To conjugate the drug, CPT was converted to CPT-Cys, and the thiol group on the Cys residue was reacted to the vinylsulfone to form the desired conjugate (Fig. 8). The CPT-PEG-LHRH conjugate had a lower IC_{50} than the CPT-PEG conjugate without LHRH peptide against A7280 human ovarian carcinoma cells.⁶⁴

C. Drug–Somatostatin Analog Peptide Conjugates

Somatostatin peptides (RC 121 and RC 160) were conjugated to DOX and 2-pyrrolino-DOX at the C14 position via a glutaric acid spacer (Fig. 9A, B). Through a series of substitution studies, it was established that the *C*- and *N*-termini residues are important for their binding affinity by stabilizing the peptide conformation. Both RC 121 and RC 160 peptides showed binding affinities to the somatostatin receptor on rat pituitary membrane homogenates in the nanomolar (below 2 nM) range. However, conjugation to DOX lowered the binding affinity to the target receptors. The effect on binding affinity may be due to interference with the peptide binding site by the drug. In in vivo studies, the conjugate of 2-pyrrolino-DOX to RC-121 had better activity and less toxicity than the drug itself in inhibiting the growth of mouse and human mammary cancers in nude mice.⁶⁵ MTX was also conjugated via its γ -carboxylic acid to the *N*-terminal of RC-121 to give MTX-RC-121 (Fig. 6C) with retained binding affinity to somatostatin receptors on rat cortex.^{27,34,66} Furthermore, MTX-RC-121 conjugate

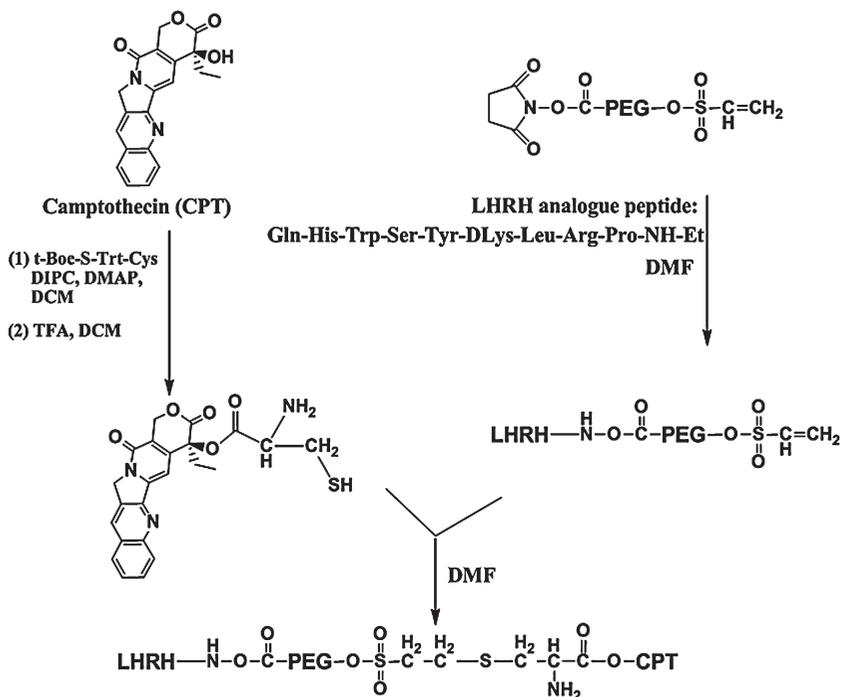


Figure 8. Camptothecin (CPT) was conjugated to an LHRH analog peptide using a polyethylene glycol (PEG) linker. CPT was conjugated to Cys to generate CPT-Cys. LHRH analog peptide was separately conjugated to NHS-PEG-VS (VS: vinylsulfone). CPT-Cys was then conjugated to LHRH-PEG-VS using thioether linking strategy (adapted from⁶⁴).

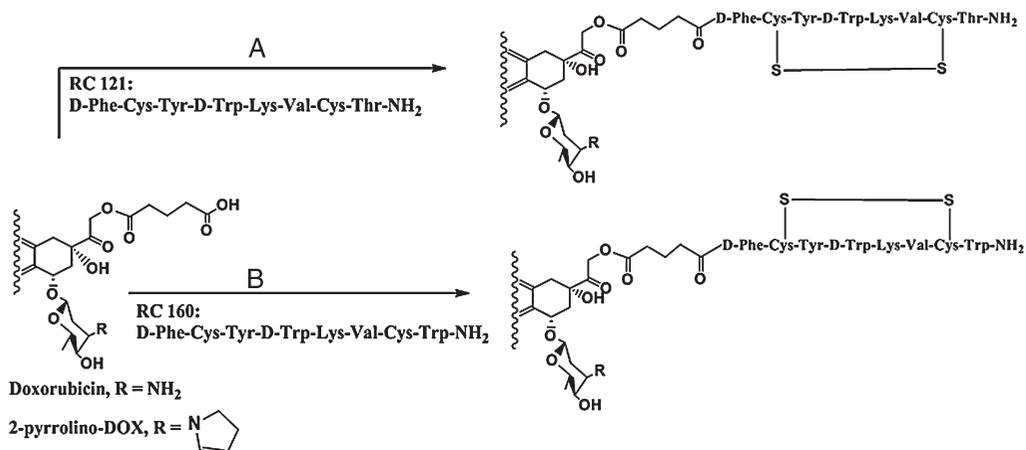


Figure 9. Somatostatin analog cyclic peptides were conjugated to DOX or 2-pyrrolino-DOX. (A) and (B) show the conjugation of *N*-termini of RC 121 and RC 160 to the C14 position of DOX and 2-pyrrolino-DOX using a glutaric acid spacer (adapted from⁶⁵).

inhibited the growth of MIA PaCa-2 human pancreatic cancer in nude mice. By contrast, neither the drug nor the peptide had an effect on inhibiting *in vivo* cancer growth.

D. Drug-RGD Peptide Conjugates

RGD peptides are the best studied peptides for targeting a specific type of cell.⁶⁷ The RGD sequence is found in many extracellular matrix (ECM) proteins, including fibronectin,

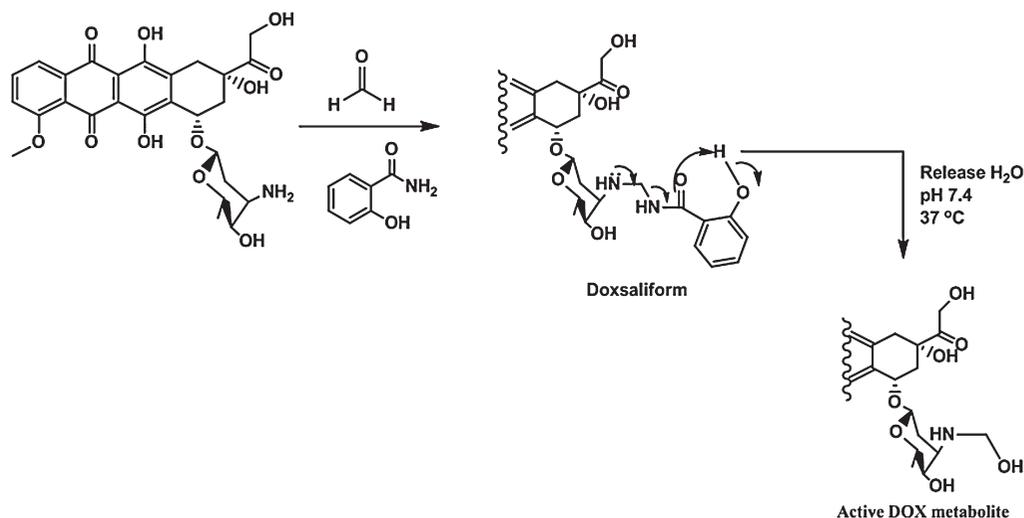


Figure 10. DOX was conjugated with formaldehyde and salicylamide to generate doxsaliform. Doxsaliform undergoes N-mannich base hydrolysis to generate the proposed active cytotoxic agent (adapted from²).

collagen, laminin, and fibrinogen. The RGD sequence is responsible for the binding of ECM proteins to the integrin family of receptors, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_5\beta_1$, $\alpha_{IIIB}\beta_3$, and $\alpha_8\beta_1$ receptors on the cell surface.^{68–71} Cyclic RGD peptides, CDCRGDCFC (RGD-4C) and cyclo-(N-Me-VRGDf), selectively bind to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which are upregulated in tumors during angiogenesis. These cyclic peptides were conjugated to a formaldehyde adduct of DOX called doxsaliform via a short hydroxylamine ether linker, which acts as a prodrug linker that can release the drug via N-Mannich-base hydrolysis (Fig. 10). The half-life of the drug release process was about 60 min, which allowed the conjugate sufficient time to accumulate in the tumors. Both drug–RGD peptide conjugates showed high binding affinity to integrin receptors in a vitronectin cell adhesion assay, and they also inhibited the growth of MDA-MB435 breast cancer *in vivo*.² In addition, paclitaxel was conjugated to a bicyclic RGD peptide ($[c(\text{RGDyK})]_2$), and the conjugate inhibited proliferation of MDA-MB435 cells. Although the conjugate had slightly lower binding affinity than the peptide itself, it had integrin-specific accumulation *in vivo*.⁷² cRGDfK peptide has been used to target liposomal nanoparticles to $\alpha_v\beta_3$ integrins on the surface of endothelial cells. This offers an attractive strategy to deliver cytotoxic drugs by encapsulating them in the nanoparticles.⁷³ Cyclic peptide analogues of c(RGDfV) with high affinity for α_v integrin receptor were conjugated to CPT analogs using both amide- and acid-sensitive hydrazide bonds. Conjugates containing acid-sensitive linker have higher *in vitro* activity and toxicity compared to the ones with stable linkers, presumably due to the greater extent of drug release from the conjugates with acid-sensitive linker. However, poor solubility of the conjugates hampered their *in vivo* evaluation. The results point out the potential challenges in optimizing the physicochemical properties of peptide–drug conjugates.⁷⁴ Overall, these results suggest that RGD peptides have promise in delivering drugs to cells with upregulated integrin receptors.

E. Drug–ICAM-1 Peptide Conjugates

Peptides derived from intercellular adhesion molecule-1 (ICAM-1) constitute a separate class of promising cell adhesion peptides that have the potential to target drugs to leukocytes. These peptides inhibited homotypic and heterotypic leukocyte adhesion mediated by leukocyte

function-associated antigen-1 (LFA-1)/ICAM-1 interactions. The LFA-1/ICAM-1-mediated leukocyte adhesion can be modulated by anti-CD11a antibodies.⁷⁵ An ICAM-1-derived cyclic peptide called cIBR (cyclo(1,12)PenPRGGSVLVTGC) binds LFA-1 via the I-domain of the α -subunit of LFA-1 and can be internalized by LFA-1-expressing leukocytes. The entry of fluorescence isothiocyanate-labeled cIBR peptide into T-cells was followed by confocal microscopy and flow cytometry.^{76–78} Therefore, cIBR peptide is an attractive molecule for targeting cytotoxic drugs to leukocytes.

To utilize cIBR peptide for targeting leukocytes, it was conjugated to MTX and DOX to give MTX-cIBR and DOX-cIBR, respectively. MTX-cIBR retained its binding affinity for LFA-1 receptors on MOLT-3 T-cells. The conjugate also showed concentration-dependent inhibition of binding of anti-LFA-1 antibody to PMA-activated MOLT-3 T-cells.⁷⁹ The MTX-cIBR conjugate was effective in inhibiting the progression of rheumatoid arthritis in the rat adjuvant model and collagen-induced arthritis mouse model. Efforts are underway to develop a stable formulation of the MTX-cIBR conjugate and to understand the *in vivo* stability of the conjugate.

The mechanism of entry of DOX-cIBR conjugate in to human leukemic cell HL-60 is via the energy-independent pathway instead of the receptor-mediated endocytosis pathway. The lack of receptor-mediated entry of DOX-cIBR conjugate is presumably due to its high hydrophobicity.²⁸ Recently, a new derivative called cIBR7 (cyclo(1,8)CPRGG SVC) has been developed that is more hydrophilic than cIBR and has higher binding affinity to the I-domain of LFA-1. However, although the DOX-cIBR7 conjugate is more hydrophilic than DOX-cIBR, the new DOX-cIBR7 conjugate still enters HL-60 cells by passive diffusion. To further increase the hydrophilicity of the conjugate, 11-amino-3,6,9-trioxaundecanoic was used to link DOX molecule to cIBR7 to form the DOX-PEG-cIBR7 conjugate, which is more hydrophilic than DOX-cIBR7. Despite the increase in hydrophilicity of DOX-PEG-cIBR7, this conjugate enters HL-60 cells via passive diffusion. Thus, it is necessary to reinvestigate the mechanisms of cellular entry of conjugates of peptides with DOX. Recently, the general applicability of ICAM-1 peptides to deliver different drug molecules with various hydrophilicities are being investigated for their selective delivery to leukocytes.

5. NOVEL DEVELOPMENTS: CLICK REACTION FOR CONJUGATION OF PEPTIDES WITH DRUGS

Using the “Click” chemistry, 1,2,3-triazoles can be easily formed via Cu^I catalyzed Huisgen 1,3-dipolar cycloaddition reaction of alkynes and azides using a wide range of temperature, pH, and solvents including water.^{80–82} Because of the regiospecificity and fast rate of the reaction in the presence of Cu^I, this reaction has been very useful for bioconjugation.⁸³ In addition, the high water solubility and rigidity of 1,2,3 triazole linker make it attractive for bioconjugation; therefore, it has been utilized to conjugate cell-penetrating peptides to nucleic acids (DNA) or peptide nucleic acids (PNA). Both alkyne-functionalized peptides and azide-functionalized PNA/DNA molecules can separately be synthesized by the solid phase peptide synthesis method. Subsequent combination of the functionalized peptides and PNA molecules was easily accomplished in water and *tert.* butanol (1:1) in the presence of CuSO₄ 5H₂O and sodium ascorbate.⁸⁴ This reaction resulted in complete conversion of the reactants to the desired product within a few hours at room temperature. Thus, this reaction can also be used to conjugate antisense/SiRNA therapeutics to cell-penetrating peptides or other peptides that can target a specific cellular receptor. However, this conjugation method can be complicated by the required addition of the functional group on the drug molecules; this added functional group may influence the efficacy of the drug.⁸¹

Despite the apparent limitations, copper catalyzed click reactions hold the potential in selective bioconjugation of peptides to drugs and oligonucleotides for delivering them to specific cells.

6. CONCLUSION

There has been increasing interest in using peptides to selectively deliver drugs to cancer cells, and promising results have been shown in both in vitro and in vivo studies. However, many aspects of drug–peptide conjugates have not been systematically elucidated, including (a) the effect of physicochemical properties of the drug on the uptake properties of the conjugate, (b) the mechanism of trafficking of the conjugate inside the cell, (c) the role of the spacer on receptor recognition and uptake of the conjugate, and (d) structural and binding properties of the conjugate vs. the peptide carrier to the cell surface receptors. There is also increasing success in targeting liposomes and nanoparticles to a specific type of cell by decorating the surface with peptides. Paclitaxel–peptide conjugates are currently undergoing phase I clinical trials for effective delivery to treat brain tumors. In the future, more data on drug–peptide conjugates will emerge to show the utility of carrier peptides in lowering the side effects of toxic drugs.

7. ABBREVIATIONS

BN	bombesin
CBT	combretastatin
CD	cluster of differentiation
CPT	camptothecin
DHFR	dihydrofolate reductase
DNR	daunorubicin
DOX	doxorubicin
ICAM-1	intercellular adhesion molecule-1
LFA-1	leukocyte function-associated antigen-1
MFBP	membrane folate binding protein
MTX	methotrexate
PSA	prostate-specific antigen
RGD	Arg-Gly-Asp

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