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Review Article

Cytotoxic conjugates of peptide hormones for cancer chemotherapy

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CONTENTS

Abstract

A major problem in traditional cancer treatment is the severe toxic side effects of chemotherapy. One approach to increasing drug specificity revolves around the receptors for certain peptide hormones that are aberrantly expressed in many human cancers and peritumoral vessels feeding tumors. This allows some peptides, when used as cytotoxin delivery vectors, to yield conjugates with specific therapeutic advantages, such as rapid tumor cell internalization and tumor-penetrating ability. Thus, peptides can be conjugated with cytotoxic agents which would concentrate in tumor tissue and/or cells through specific peptide-receptor interactions. Overall, this relatively new approach to chemotherapy effectively increases the specificity and efficacy of the cytotoxic agent and should decrease toxic side effects. Peptide hormones such as somatostatin (SST), bombesin (BN), luteinizing hormone-releasing hormone (LHRH) or their analogues have been used as drug delivery vectors when linked with various cytotoxic agents, such as camptothecin (CPT), paclitaxel (PTX) and doxorubicin (DOX). Several lead cytotoxic peptide conjugates have been developed and demonstrate greater cytotoxicity against various cancers together with reduced toxicity to normal tissues. Two examples, JF-10-81 and AN-152, are currently undergoing preclinical and clinical development, respectively. These new cytotoxic peptide conjugates may provide more effective chemotherapy and drug delivery protocols for receptor-specific cancer treatment in the near future.

Introduction

One of the major problems associated with the use of potent cytotoxic drugs in cancer chemotherapy is the lack of selectivity for cancer cells relative to normal cells. The resulting damage to normal cells and tissues can result in severe toxic side effects, thus limiting the effective dose of the anticancer compound. Perhaps the ideal anticancer agent would be a “magic bullet”, an idea conceived by Ehrlich over 100 years ago (1), that would specifically recognize only cancer cells before destroying them (1, 2). One approach has utilized monoclonal antibody (MAb) technology developed by Kohler and Milstein in 1975 (3), which takes advantage of the prolific expression of specific antigenic sites on tumor cells and antibodies raised against them.

Cytotoxic MAb conjugates were prepared by attaching toxins to MAbs and several were then demonstrated to have improved anticancer activity against various cancers when suitable cleavable linking groups were employed (4-8). More recently, another kind of important targeting strategy involving short peptide hormones has been utilized. Initially, this technique was used to couple a variety of radionuclides for both radiotherapy and imaging applications (9, 10). This was extended to chemotherapeutic peptide conjugates first employing the cytotoxic agents chlorambucil and melphalan coupled to luteinizing hormone-releasing hormone (LHRH) analogues (11, 12). Subsequently, peptides as vectors coupled with chemical agents have been shown to be a relatively sophisticated approach to increasing cellular uptake, improving efficacy and reducing toxic side effects (13-16).

In our laboratory, we have been using suitable peptide candidates, such as somatostatin (SST) and bombesin (BN), conjugated with various other cytotoxic compounds. For this approach to be effective, it is desirable to use linking chemistries between the peptide and drug that promote the specific cleavage and release of the active component into the tumor tissue or, ideally, within the tumor cell itself. This has been the subject of many synthetic chemistry studies which have been extensively reviewed (17, 18). In several instances, novel peptide conjugates have been demonstrated to target receptor-

specific cancer cells with greater *in vivo* efficacy than the original compounds themselves (16, 19, 20).

Peptide hormones

Many peptide hormones are relatively small molecules compared to large-molecule proteins such as antibodies. Various peptides exist in the human body that are readily metabolized and cleared from the body. This is a major disadvantage, although analogues can be synthesized with greater stability and, in some instances, even higher affinity than the natural substances (21). Certain peptide hormones, such as SST, BN, LHRH, pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP), or their analogues, can be utilized as potential vectors for cytotoxic agents since their receptors are aberrantly expressed at higher levels in cancer cells than in normal cells and in active endothelial cells *versus* quiescent cells. Thus, these peptides or their analogues can deliver and internalize (in the case of agonists) cytotoxic agents to receptor-targeted sites (16, 20-23).

In addition, peptides as vectors have certain other advantages because they are easy to synthesize and optimize and can be quickly investigated for their therapeutic potential. Also, peptides are small molecules which rapidly internalize, are rapidly cleared from the circulation and have a good tumor tissue-penetrating ability. Peptides also should not stimulate the body's immune response or cross the blood-brain barrier (21). Peptides can be readily conjugated with various compounds, with each portion retaining its biological functions as long as suitable linking chemistries can be devised. Stable peptide analogues can be made to compensate for the short half-life of natural peptides, and for these reasons peptide-based targeted therapy has become a novel alternative with promising therapeutic potential (15, 21, 24, 25).

Cytotoxic SST conjugates

The SST family consists of two naturally occurring members, SRIF-14 and SRIF-28, and these interact with five somatostatin receptor (SSTR) subtypes present in varying ratios on many different cells and tissues. Both native SSTs bind with high affinity to all SSTR subtypes and all these SSTRs, particularly the sst_2 (SSTR2) subtype, are highly expressed or even overexpressed in a variety of tumors, including lung, breast, prostate and gastrointestinal tract tumors (21, 26-28), and in the peritumoral vessels of many tumors, thus offering a generic target (20, 21, 29, 30). Not only are the SSTs and their analogues rapidly internalized into cells, but they may also be translocated to the cell nucleus (20, 29, 30). Additionally, SST analogues can exhibit differential high-affinity binding to particular receptor subtypes, allowing precise targeting of an SST analogue carrying an effective cytotoxic molecule to specific diseased tissues. We have developed other analogues which bind to all five receptors when conjugated to camptothecin (CPT), thus potentially yielding a conjugate capable of targeting most tumor types, which should be particularly valuable for use in patients whose receptor profiles are not known. In summary, SST analogues and SSTRs can be considered desirable targeting agents because of the receptor expression profile and also the fact that the peptides themselves display few toxic side effects in humans.

As mentioned above, in our laboratory we have coupled the chemotherapeutic agent CPT to the *N*-terminus of an SST analogue via a cleavable carbamate group, the stability of which can be adjusted by incorporating variable built-in nucleophile assistance, and an *N*-terminal linking motif, D-Ser-Nle-D-Tyr-D-Lys (16). This motif allows the attachment of large groups to the *N*-terminus of SST octapeptide analogues without loss of sst_2 affinity (15, 16). A series of sst_2-specific CPT-SST conjugates was prepared and JF-10-81 was selected as our lead therapeutic candidate. This conjugate is very stable in plasma (half-life = 18 h) compared to native SST-14 (half-life of several minutes), readily water-soluble and should be cleaved by intracellular enzymes of the cytochrome P-450 family, thereby selectively releasing CPT within SSTR-expressing tumors or peritumoral vessels. JF-10-81 reduces the toxic side effects associated with naked cytotoxins and is in preclinical development as a potential treatment for abnormal angiogenesis associated with age-related macular degeneration (AMD) (13).

JF-10-81 has been shown to selectively bind to sst_2 receptors and exert potent inhibitory activity against various tumor cells in *in vitro* experiments (16, 31). In *in vivo* experiments, JF-10-81 exhibited receptor-positive tumor-targeting ability by significantly inhibiting the growth of rat pancreatic CA20948 tumors and human small cell lung cancer (SCLC) NCI-H69 tumors, while having no effect on human prostate cancer PC-3 tumor growth at a less frequent low dose (16). However, at a more frequent higher dose, JF-10-81 showed significant inhibition of PC-3 tumor growth, resulting in almost 60% reduction of tumor weight relative to untreated animals (unpublished data). Furthermore, by using continuous drug-releasing pellets (5 mg/pellet), JF-10-81 was found to have much more potent inhibitory activity against SSTR-positive and CPT-sensitive tumors. JF-10-81 resulted in > 90% reduction of human neuroblastoma IMR-32 and leukemia MOLT-4 tumors, even degenerating human pancreatic cancer CFPAC-1 tumors. In nude mice bearing SSTR-overexpressing but CPT-insensitive pancreatic carcinoid BON cells, the conjugate resulted in > 90% reduction of tumor volume, although the tumor cells themselves were relatively resistant to CPT *in vitro* (unpublished data). Furthermore, sustained delivery of JF-10-81 showed more inhibitory activity than CPT itself. Interestingly, the conjugate also demonstrated antiangiogenic activity and inhibited PC-3 tumor cell invasion *in vitro* and tumor metastasis (> 70% reduction) *in vivo* (unpublished data). A possible signaling pathway involving integrin $\alpha_v\beta_3/\alpha_v\beta_5$, the metalloproteinases MMP-2/9 and phosphatidylinositol 3-kinase (PI3K)/Akt was implicated (32). The conjugate

was also a potent inhibitor of abnormal laser-induced blood vessel growth in a rat model of AMD (13).

Paclitaxel (PTX, Taxol®) also shows potent antitumor activity in a wide variety of tumor models. PTX was conjugated to the *N*-terminus of the octapeptide SST analogue octreotide and appeared to be exclusively cytotoxic for SSTR-expressing breast carcinoma MCF7 cells, with much less toxicity against SSTR-negative CHO cells (33). MTX was also coupled to another octapeptide, the sst_2-specific SST analogue RC-121, and the conjugate showed inhibitory activity against human pancreatic cancer xenografts in nude mice, although this was not significant (34).

Another potent cytotoxic molecule, doxorubicin (DOX), or its derivative 2-pyrrolino-DOX, which had over 100-fold greater activity than DOX itself (35), was conjugated to octapeptide sst_2-specific SST analogues. AN-238 was prepared by coupling 2-pyrrolino-DOX to the SST analogue RC-121 via the less than ideal ester linkage and appeared to be the most effective conjugate, binding preferentially to sst_2 and to a lesser extent to sst_5 (36, 37). AN-238 retained high SSTR binding affinity and had potent cytotoxicity (38). It could also inhibit the growth of various SSTR-positive tumors as a single low dose (37, 39), and, despite the abundance of esterases in the circulation and healthy peripheral tissues, it appeared to be more effective and less toxic than 2-pyrrolino-DOX itself (21, 40). In addition, AN-238 demonstrated indirect antitumor activity against human SSTR-negative non-SCLC NCI-H157 xenografts by directly targeting SSTR-positive tumor blood vessels of the host mice (41). AN-238 has been reviewed elsewhere (37, 38).

Cytotoxic BN conjugates

Bombesin (BN) is another relatively short-sequence neurogastrointestinal peptide that can be easily synthesized in the laboratory or on a commercial scale. There are four different subtypes of BN receptors, including the neuromedin B receptor (NMB-R or BB1), the GRP receptor (GRP-R or BB2), the BN receptor subtype 3 (BRS-3 or BB3) and the nonmammalian BN subtype 4 (BB4) (42, 43). Except for GRP-R, all of the other subtypes have been less well studied in human tumor tissues; however, the human GRP-R is expressed abundantly in different types of cancer, such as gastric, colon, ovarian, prostate, lung and breast cancer, but has limited distribution in normal tissues (21, 43-51). Although GRP-R is rarely expressed in pancreatic cancer cells, it is clearly expressed in tumor vessels surrounding pancreatic cancers (21). As with SST peptides, the specific characteristics described above suggest that BN and its receptors might be used for cancer-targeting therapeutics.

In our laboratory, a CPT-BN conjugate (DC-51-43 or CPT-L2-BA3) was prepared by linking CPT to a BN(4-14) agonist analogue via a built-in nucleophile-assisted release (BINAR) carbamate linker. This conjugate bound to NMB-R, GRP-R and BRS-3 receptors with high affinity and underwent rapid internalization into GRP-R-transfected CHO cells. A control analogue, D-Phe-CPT-L2-BA3, was also prepared which had very little affinity for BN receptors (52). The active agonist CPT-L2-BA3 inhibited the growth of cultured tumor cells, including the most common BN receptor-expressing cancer cells, such as prostate, breast, lung, colorectal and pancreatic cancers (32, 53), displaying significantly more cytotoxicity than the inactive D-Phe-CPT-L2-BA3 against NCI-H1299 lung cancer cell growth. CPT-L2-BA3 also inhibited the growth of pancreatic, SCLC and prostate tumors in nude mice (53). CPT-L2-BA3 has also demonstrated antiangiogenic activity in several experiments (32).

In other related studies, a BN analogue was conjugated to PTX using a hetero-bifunctional polyethylene glycol (PEG) linker. Compared to free PTX, this new conjugate, PTX-PEG-BBN[7-13], maintained receptor binding affinity and was more effective against receptor-positive non-SCLC NCI-H1299 cells (52). The conjugate appeared to improve PTX cytotoxicity (54). In an extension of this approach, two BN peptide molecules were conjugated with one PTX molecule. This new multiligand conjugate showed greater cytotoxic activity than both free PTX and the monoligand conjugate (55). The multiligand approach is interesting and may potentially become a promising new avenue of investigation (55, 56). In these PTX-BN conjugates, PEG enhanced solubility after being coupled; however, the cytotoxicity decreased if too many PEG molecules were employed (54, 55).

In another study, a BN analogue was linked with an MAb and the new peptide-antibody conjugate stimulated T-cell activation and increased cytotoxicity against SCLC cells (57, 58). Another BN conjugate, AN-215, which was synthesized by linking 2-pyrrolino-DOX to the *N*-terminus of a BN(7-14) analogue through an ester linkage to a glutaric acid spacer, showed high affinity for GRP-R and significant inhibition of various tumors (38, 59). However, the ester linkage has been reported to be too stable after internalization while being rapidly metabolized in peripheral tissue (60).

Cytotoxic VIP/PACAP conjugates

The VIP and PACAP peptides belong to the secretin/glucagon family of neurogastrointestinal peptides. There are three VIP/PACAP receptors, named $VPAC_1$, $VPAC_2$ and PAC_1. Naturally occurring $PACAP_{1-27}$ and $PACAP_{1-38}$ bind with high affinity to all three receptors, whereas VIP binds with high affinity only to $VPAC_1$ and $VPAC_2$. These three receptors are expressed in the majority of the most frequently occurring human cancers, including breast, prostate, pancreatic and bladder cancer. Indeed, most primary human tumors appear to express VIP/PACAP receptors in high density (21, 29, 61). We prepared a VIP-CPT conjugate,VIP-L2-CPT, by coupling CPT to a VIP analogue through a BINAR carbamate linker to a Lys side-chain in position 29. The VIP conjugate lost some binding affinity, but it could be effectively internalized by breast cancer MCF7 cells and inhibited cell proliferation (62). Additional cytotoxic VIP/PACAP conju-

gates have been prepared in our laboratory and a number of them retain the high binding affinity of VIP/PACAP, have significant inhibitory activity against tumor cell proliferation (unpublished data) and are under investigation.

Cytotoxic LHRH conjugates

LHRH is a decapeptide hormone synthesized and released by the hypothalamus and responsible for the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. Interactions between LHRH and its receptors appear to result in receptor microaggregation and internalization of the peptide (37). LHRH receptors are expressed in many human cancers, such as breast, ovarian, prostate and pancreatic cancer (35, 37, 59), and their expression is higher in various cancers than in normal tissues (35). This potentially provides an advantageous use of LHRH or its analogues as drug delivery vectors (11, 12, 35, 37, 63). DOX was esterified to an LHRH analogue via a D-Lys^6 side-chain to give AN-152. 2-Pyrrolino-DOX was also coupled to give the analogue AN-207. Both of these LHRH conjugates bound to LHRH receptors with high affinity (37, 64) and showed excellent antitumor profiles. AN-152 is in clinical trials and recently successfully completed a phase I trial in the treatment of gynecological and breast cancers. The investigation and clinical progress of the compound have been reviewed (35, 37, 38, 59, 64).

Other conjugates

The examples given above represent a subset of the most popular peptides that have been used as vectors and types of cytotoxins used in conjugates. There are, however, less readily classifiable examples such as the "vectocell" peptide used as an intracellular delivery tool and a driver of the attached drug across the cell membrane. This peptide was linked to DOX and the new vectocell conjugate improved antitumor efficacy in colon and breast cancer (65). In another example, PTX was linked with the erbB-2-recognizing peptide EC-1 and selectively inhibited erbB-2-overexpressing human breast cancer cell proliferation (66). The MMP-specific peptide-DOX conjugate had potent inhibitory activity against tumor growth and reduced toxicity (67). Additionally, neuropeptide Y (NPY) was used to deliver daunorubicin to neuroblastoma SK-N-MC cells selectively expressing the NPY Y_1 receptor. This conjugate showed specific cytotoxicity, while it had no cytotoxicity against NPY receptor-negative glioblastoma XF-498L cells, indicating selective tumor-targeting ability. There is evidence that NPY actually delivers compounds to the cellular nucleus after internalization, while an inactive, nonbinding peptide conjugate did not (68).

Peptides as vectors are synthetically very flexible and can be attached to many different groups. They can also be readily linked with a second peptide to form heterobifunctional peptide-peptide conjugates (69, 70), and also with antibodies (57, 58), antisense oligonucleotides or peptide nucleic acids (19, 71) and small interfering RNAs (siRNAs) (72). These different conjugates have shown anticancer activity and selectivity at different levels and may be more powerful due to additional receptor interactions.

Conclusions

Increasing the specificity and efficacy of cancer therapy using targeted vector approaches has long been a dream in cancer chemotherapy and has been realized mostly using antibody targeting. Several antibody conjugates are widely used in cancer treatment. However, antibodies are very large proteins and can be nonspecifically taken up by the liver and the reticuloendothelial system. Also, antibodies have difficulty penetrating entire tumors and thus have high toxicity (24, 25). Peptide hormones and their more stable synthetic analogues are an excellent, although less-investigated alternative and are possibly more effective for specifically targeting receptor-expressing cancers and may also avoid some of the problems associated with antibody targeting (25, 37).

As described above, internalizing receptors for a number of peptide hormones are aberrantly expressed in many human cancers and thus a wide variety of receptor-targeting cytotoxic peptide conjugates can be envisaged. The peptides mentioned above were used as drug delivery vectors because they are dramatically flexible from a chemical viewpoint, with the ability to tolerate linking to various cytotoxic groups with retention of their functional activities. Radiolabeled peptide therapy has become a very promising approach for tumor treatment and imaging. However, one problem is dose limitation due to the fact that the radiation dose may potentially destroy receptor-positive normal tissues, such as the immune system and vital organs such as the kidney and liver, which are responsible for the excretion and elimination of the radiopeptide conjugates not bound to the targeted tumors (21).

It is already apparent that novel peptide conjugates can increase a compound's cancer cell selectivity and cytotoxicity and decrease toxicity to normal cells. It is only a question of time before the first of these new conjugates receives FDA approval. We can look forward to additional improvements to the method, particularly in the development of more specific linking strategies and new drug delivery methods, such as sustained delivery formulations, which take advantage of decreased toxicity relative to traditional chemotherapeutic agents.

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