Research and Reviews: Drug Delivery

Cytotoxic Peptide Conjugates: Anticancer Therapeutic Strategies

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ABSTRACT

Traditional chemotherapeutic agents exhibit potent anticancer efficacy. However, in clinical applications, they also exhibit severely toxic side effects, and result in multidrug resistance (MDR) of cancer cells. So, receptor-targeted therapy is catching more attention of scientists from both academic and industry and recently is coming to the central stage of drug development. Certain peptides, due to their advantages like, easy synthesis and low cost, less or no immunogenicity, stability and high affinity, have been used as drug delivery vehicles. For example, cell-targeting peptides and cell-penetrating peptides (CPPs) in conjugation with cytotoxic agents have elicited remarkable effects. Luteinizing hormone-releasing hormone, somatostatin, and bombesin/gastrin-releasing peptide are the cell-targeting peptides that interact with their cognate surface receptors aberrantly expressed in many cancer cells, so these hormone peptides can be incorporated into cytotoxic agents for cell-specific targeting in cancer chemotherapy. Due to their cell-penetrating ability, CPPs also serve as cytotoxic drug delivery vehicles to carry drugs across the plasma membrane and overcome MDR of cancer cells. Cytotoxic agents linked to cell-targeting peptides and CPPs have been considered as an effective and reliable method in cancer chemotherapy. In this review, we address the applications of these peptides as drug delivery vehicles in targeted anticancer drug development.

INTRODUCTION

Chemotherapy is a primary modality to treat advanced or metastatic cancers. However, traditional chemotherapeutics are restricted by the acquired or intrinsic multidrug resistance (MDR) of cancer cells and toxic side effects that are caused by high drug doses necessary to achieve treatment efficacy [1,2]. Some drugs such as taxol are poorly soluble and thus should be formulated in organic solvents. However, this procedure may cause allergies. Various drug systems have also been investigated. Tumor cells overexpress various receptors and biomarkers, which can be used as targets to deliver cytotoxic agents into tumors [3]. Several tumor-recognition moieties such as monoclonal antibodies (mAbs), hyaluronic acid, folic acid, and peptides have been identified for their tumor-targeted specificity. One strategy for synthetic tumor targeting drugs is the use of tumor-recognition moieties conjugated with anticancer agents. Cytotoxic antibody-drug conjugates (ADCs) are prepared by attaching cytotoxins to mAbs. These conjugates exhibit improved anticancer activity against various cancers when suitable and cleavable linking groups are used [4-8]. Despite high target specificity, the high molecular weight of mAbs is a major drawback [9]. Furthermore, mAbs are characterized by low tissue penetration and poor cellular uptake when these antibodies are used *in vivo* [10]. Polypeptides that specifically recognize tumor cells via interacting with cell surface receptors and show high cellular uptake can overcome these limitations. In contrast to mAbs, polypeptides exhibit almost negligible immunogenicity and likely cause minimal side effects. For almost 20 years, the use of drugs in conjugation with cell-targeting peptides or cell-penetrating peptides (CPPs) has been a novel strategy to deliver non-specific anticancer cytotoxic agents.

After detecting the specificity of antigen-antibody interactions, Paul Ehrlich proposed the concept of a 'magic bullet' for cancer therapy. The 'magic bullet' can specifically target and destroy cancer cells, not hurting normal cells with less toxic side effects. One of such therapeutic strategies is to link cytotoxic agents to tumor cell-targeted molecules [11]. In 1970s, the technology had been developed to generate monoclonal antibody (mAb) that was applied to target specific tumor cells. Subsequently, the discovery of peptide receptors such as the receptors of luteinizing hormone-releasing hormone (LHRH), somatostatin (SST), and bombesin (BN)/gastrin-releasing peptide (GRP), which are highly overexpressed in many tumor cells, provided another approach to deliver non-specific drugs to target sites via coupling the drugs to receptor-binding peptide vehicles. These new products are the so-called receptor-targeted peptide-drug conjugates.

CPPs, a class of small (<20 amino acid) cationic peptides, can facilitate the uptake of large biologically active molecules into mammalian cells [12]. The most frequently used CPPs include Tat, oligoarginine, and antennapedia (Antp). The bio-distribution of CPPs and their conjugates with bioactive payloads suggests their preferential accumulation in the liver, kidney, lung, and spleen [13-18]. CPPs are also used as drug delivery vehicles because of their excellent ability to facilitate cell uptake and overcome MDR although they exhibit less specificity than receptor-targeting peptides. In this review, we will address the use of both receptor-targeted peptides and cell-penetrating peptides (CPPs) as drug delivery vehicles.

MECHANISMS OF PEPTIDE ENTRY INTO CELLS

As drug delivery vehicles, both receptor-targeted peptides and CPPs display different mechanisms in delivering drugs into targeted tumor sites. The hydrophobic nature of the cell membranes provides a significant barrier against peptide entry into cells. However, receptor-targeted peptides can penetrate the cell membrane through receptor-mediated endocytosis via active transport. Thus, anticancer agents can be conjugated with these peptides, which function as ligands that bind to their cognate receptors and induce energy-dependent endosome formation. Endosomes then enter cells and become degraded by lysosomes. Afterward, drugs are released into the cytoplasm **(Figure 1).**

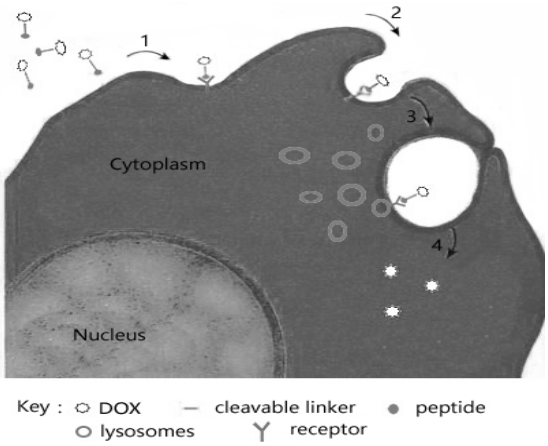

Figure 1. Schematic of the receptor-mediated entry of cytotoxic peptide drugs into cancer cells. 1 cytotoxic peptide drug-specific recognition of a cell surface receptor; 2 endosome formation; 3 endosome-lysosome fusion; 4 endosomal escape and delivery of drugs to the cytoplasm and then to the nucleus.

As for CPPs, the mechanism of their entry into cells remains unknown although being extensively investigated. As reported, the uptake of these peptides likely occurs efficiently at 37 °C, but endocytosis unlikely happens [19-21]. The internalization of CPPs is considered as a passive process. In general, a peptide-drug conjugate interacts with and moves across the cell membrane. Drugs are then released inside the targeted cells **(Figure 2).**

CPPs are transduced through macropinocytosis, which is a specialized form of endocytosis. This discovery has created a new paradigm in the new study of peptide-drug conjugates [22]. The entry of CPP conjugates into cells is demonstrated using a multi-step model. In this model **Figure 2B**, CPPs initially bind to the cell membrane, subsequently with macropinocytosis stimulated. Macropinosomes undergo peptide or cargo uptake. Afterward, endosomes escape into the cytoplasm. First, peptides bind to the plasma membrane through electrostatic interactions with sulfated glycan's such as heparin sulfate, hydrophilic component of the phospholipid bilayer, and acid regions of proteins. As a consequence, the membrane becomes negatively charged and may be responsible for macropinocytosis and membrane transduction. However, the exact mechanism by which CPP conjugates bind to a cell surface to facilitate uptake remains unknown. Next, micropinocytosis occurs and it's a form of fluid phase endocytosis wherein actin protrusions fold in on the cell and uptake the surrounding medium. Nevertheless, the specific mechanism by which macropinocytosis is stimulated by CPPs remains unclear. Eventually, drugs are released into the cytoplasm by macropinosomes. This process is also considered as a major limitation of CPP-based drug delivery [23]. Several components such as lytic peptides [24-26], pH-sensitive polymers [27] or swellable dendritic polymers [28] have been developed to enhance endosomal escape (peptides escape from endosomes. Fischer et al. reported that CPPs can travel through the endoplasmic reticulum and the Golgi network via a 'retrograde pathway' that involves cytosolic release [29]. We discussed the applications and advances of cell-targeting peptides and CPPs as drug delivery vehicles in the treatments of cancers.

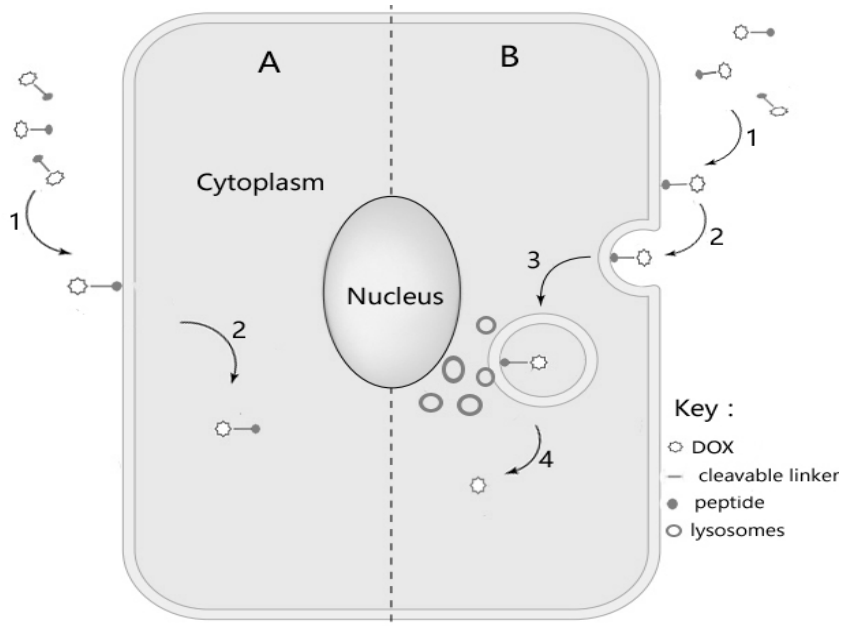

Figure 2. Transduction (A) and macropinocytosis (B) of CPP-drug conjugates. (A) Transduction: 1) binding of a cytotoxic CPP drug conjugates to the cell surface via a cell surface proteoglycan platform; 2) directly transporting across the cell membrane and releasing the drugs. (B) Macropinocytosis: 1) binding of CPP drug conjugates to the cell membrane; 2) Formation of macropinosome complex; 3) degradation of the macropinosome complex by lysosomal enzymes; 4:escaping lysosomal degradation and entry into the cytosol or nucleus.

CELL-TARGETING PEPTIDES AS CYTOTOXIC DRUG DELIVERY VEHICLES

As described above, some peptide receptors have been discovered over the last five decades. Certain of these receptors can be utilized as potential drug targets in chemotherapy [30-34], with their cognate ligands being peptides that can be conjugated with cytotoxic radicals for drug delivery to cancer cells expressing the corresponding receptors. Cytotoxic agents have been coupled to the receptor-specific analogs of these hormonal peptides such as LHRH, SST and BN/GRP. These new peptide-drug conjugates can target to certain cancer cells possessing receptors via ligand-receptor interactions. Consequently, the receptor-selectivity enhances the efficacy of drugs against cancer cells.

Cytotoxic LHRH Drug Conjugate

LHRH and its receptors

LHRH, also known as the gonadotropin-releasing hormone (GnRH), is a hormonal decapeptide produced by the hypothalamus; the hormone plays a key role in the regulation of the pituitary/gonadal axis and reproduction [35]. Sex steroids have been involved in the development of breast, ovarian and prostate cancers; thus, the agonistic analogs of LHRH have been widely used in phymatology and gynecology for nearly four decades [36,37]. Compared with that in cancer cells, LHRH receptors are only expressed at low levels in healthy cells of organs such as the ovary, prostate, and testes. These receptors are not present in most normal tissues [38,39]. High-affinity binding sites for LHRH and the expression of mRNA for LHRH receptors were detected in human prostate, breast, epithelial ovarian and endometrial cancer cells. LHRH receptors are present in 86% of human prostate cancers [40], more than 50% of estrogen-negative breast cancers [41], 78% of ovarian cancers [42,43], and nearly 80% of endometrial cancers [44]. In addition to the reproductive organ cancers, LHRH has been detected in the cancers of some non-reproductive organs that are affected by the pituitary/gonadal axis such as the oral and laryngeal cancers, brain cancers, liver cancers, renal cancers, adenocarcinomas of the colon, melanomas and ductal pancreatic carcinomas [1].

Anticancer agents linked to LHRH analogs

The aberrant expression of LHRH receptors in various cancers provides fundamental information for the design and synthesis of cytotoxic LHRH drug conjugates. Natural LHRH has a very short half-life *in vivo*. Thus, the substitution of a Gly amino acid (aa) residue at position 6 of LHRH by various d-aa residues produce highly potent and stable LHRH analogs with high binding affinity. Numerous experiments have shown that a d-Lys moiety at position 6 can offer an amino side-chain for the covalent link of various cytotoxic radicals. Furthermore, the introduction of a d-aa can extend its half-life *in vivo*. An early cytotoxic LHRH drug conjugate consists of [d-Lys6] LHRH linked to the daunosamine nitrogen moiety of doxorubicin (DOX) by a glutaric acid spacer [45]. Unfortunately, the anti-proliferative activity of DOX is severely lost. The 14-O ester bonds of DOX are stable and this modified DOX have similar anticancer activity as DOX. Thus, the DOX-14-O-hemiglutarate was conjugated to [d-Lys6] LHRH to form the cytotoxic LHRH drug conjugate AN-152 (AEZS 108; **Figure 3**) [46]. A daunosamine-modified derivative of DOX, or 2-pyrrolino-DOX (AN-201), is 500 to 1000 times more active *in vitro* and *in vivo* than its parent compound. In addition, AN-201 is neither cross-resistant nor cardiotoxic with DOX [47]. The super-active cytotoxic LHRH drug conjugate AN-207 was prepared from AN-152 via the same

synthetic strategy [48]. AN-207 and AN-152 have been investigated *in vivo* with various tumors and have proven their antitumor efficacy **(Figure 3).**

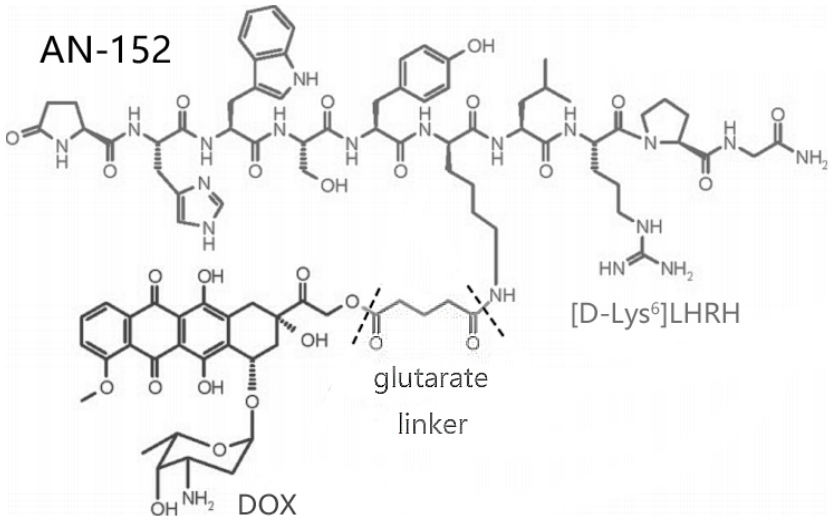

Figure 3. Molecular structure of the cytotoxic LHRH drug conjugate AN-152, which consists of [d-Lys6] LHRH covalently linked to DOX by a glutaric acid spacer.

Antitumor evaluation of cytotoxic LHRH drug conjugates

Two cytotoxic LHRH drug conjugates AN-152 and AN-207 have shown excellent antitumor efficacy and low toxic side effects in numerous preclinical *in vivo* models of tumors grown from LHRH receptor-positive cancers. AN-152 and AN-207 were tested in nude mice bearing xenografts of human breast cancer cells, including the DOX-resistant and LHRH receptor-positive human MX-1 breast cancer cells. Both conjugates strongly inhibited the growth of tumors whereas the unconjugated DOX was less effective. In nude mice bearing estrogen-independent MXT mouse breast cancer cells, AN-152 produced approximately 90% inhibition of tumor growth. AN-207 at a 100-fold lower dose achieved the same effect. Furthermore, AN-207 induced the major reduction of tumors from the estrogen-independent human breast cancer MDA-MB-231 cells [49]. In another investigation [50], nude mice bearing tumors from HCC1806 and MDA-MB-231 human triple-negative breast cancer cells were injected with the conjugate AN-152. AN-152 showed complete inhibition of tumor growth. A phase I study [51] further showed that AN-152 at a dose of 267 mg/m^2 and at three-week intervals was optimal. AN-207 is more advantageous. AN-207 at a dose that is 150-200 times lower than that of AN-152 can achieve the same antitumor effects [52]. The given doses and schedules of AN-152 and AN-207 had no significant and long-lasting effects on the hormonal activity, with the estradiol levels and sex organ weight not decreased.

The conjugate AN-152 also showed its effects on the growth of LHRH receptor-positive OV-1063 ovarian cancer tumors, but not on the LHRH receptor-negative UCI-107 human epithelial ovarian cancer tumors. Meanwhile, AN-152 also inhibited the growth of LHRH receptor-positive OVCAR-3 ovarian cancer tumors. AN-152 and AN-207 were investigated in LHRH receptor-positive ES-2 ovarian cancer tumors in nude mice [53]. Subsequent studies demonstrated that the growth of tumors from human ovarian cancer ES-2 and OV-1063 cells was inhibited by AN-207 at a dose that is 100 times lower than that of AN-152 [53]. A total of 43 patients with taxane-pretreated and platinum-resistant receptor-positive human ovarian cancer were included in a phase II study [54]. The patients received up to 6 cycles of AN-152 at dose of 267 mg/m^2. A total of five patients (11.6%) in partial remission and fourteen patients (32.6%) in stable condition for more than 12 weeks were achieved. The median time to progression (TTP) was determined to be 3.5 months, and the median overall survival (OS) was 15 months [55].

The antitumor efficacy of AN-152 was demonstrated in the RL-95-2, HEC-1A and HEC-1B human endometrial cancers xenografted into nude mice. After injection of AN-152 at a dose of 15 μmol/kg once weekly, the HEC-1B tumor volume was reduced by approximately 25%; at a dose of 17 μmol/kg, AN-152 can significantly inhibit tumor growth [56]. The phase II study showed that the median TTP is 7 months, and the OS is 14.3 months [57]. In the same study, AN-207 significantly inhibited the growth of RL-95-2 and HEC-1A human endometrial cancer xenografts in nude mice.

In another case, the cytotoxic conjugate AN-152 inhibited the growth of androgen-independent intra-osseous C4-2 prostate cancer tumors and also decreased the serum prostate-specific antigen (PSA) levels [58]. The conjugate can also inhibit the growth of the androgen-sensitive MDA-PCa-2b and LNCaP prostate cancer tumors. In Dunning R-3327-H or androgen-independent R-3327-AT-1 human prostate cancers xenografted into nude mice, AN-207 induced the major reduction of the tumor volume, whereas AN-201 at the same dose had no effect. In nude mice bearing PC-82 human prostate cancer tumors, significant inhibition was observed after the administration of AN-207 [59]. AN-207 also significantly inhibited the growth of MDA-PCa-2b human prostate cancer tumors and decreased the PSA levels [60].

Cytotoxic SST Drug Conjugates

SST and its receptors

Somatostatin (SST) is also known as the somatotropin release-inhibiting factor (SRIF). SST was first discovered in the sheep hypothalamus, and its tetradecapeptide structure was demonstrated in 1973 by Brazeau et al. [61]. Subsequently, another SST isotype (SST-28) was isolated from the pig hypothalamus; this variant was an extension of SST-14 at the N-terminus. Both SSTs affect cell proliferation, endocrine secretion, and neurotransmission by activating its cognate receptors, thereby demonstrating the potential to modulate various pathological conditions [62]. The five SST receptor subtypes (SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5) belong to the family of G protein-coupled receptors (GPCRs). SSTR2 has two spliced variants, SSTR2a and SSTR2b. These receptors have high affinities to SST and its synthetic analogs and are expressed at significantly elevated levels in various cancer cells, including neuroendocrine tumors (NETs) (pancreas, pituitary, lung, gastrointestinal, medullary, prostate, and bone cancers, etc.) and non-NETs (ovarian, lung, breast, cervical, colorectal cancers, etc.) [62]. Among these receptors, SSTR4 is less expressed in the brain [63]. SSTR2 is highly expressed in tumors and angiogenic blood vessels [64], especially in proliferative endothelial cells, not in non-proliferative ones. Thus, SSTR2 was applied as a target for drug development.

Functions of SST and its analogs

The anti-proliferative effects of SSTs could be direct or indirect [65]. The major function of SSTs is to induce cell growth arrest, anti-angiogensis, and tumor growth suppression via direct mechanisms by inducing apoptosis and inhibiting cell proliferation. Another major function of SSTs is to suppress growth hormone release, which in turn leads to reduction of insulin-like growth factor-I (IGF-I) release. The decreased level of IGF-I could inhibit the growth of various tumors. The extremely short half-life of SSTs *in vivo* limits its clinical applications. Thus, serial SST analogs were synthesized by shortening the SST sequence and introducing d-amino acids instead of l-amino acids. Some of these analogs including lanreotide, octreotide, pasireotide, vapreotide, seglitide and DG3173, have been approved by FDA and introduced in the market. Furthermore, SST analogs have been used as cytotoxic drug delivery vehicles in the development of peptide-drug conjugates and for radio-isotopes-peptide imaging.

Anti-tumor evaluation of cytotoxic SST drug conjugates

SSTRs, particularly SSTR2 subtype, are highly expressed in many cancers such as pancreatic cancer, lung cancer, neuroblastoma. This provides a great opportunity for developing SSTR-targeted anti-cancer drugs **(Figure 4)**. Therefore, various cytotoxic SST-drug conjugates have been developed and demonstrated for their potent specific anti-tumor activities. The potent and cytotoxic 2-pyrroline-DOX (AN-201) was conjugated to the octapeptide SST analog RC-121 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH2) via the α-amino group of its N-terminal D-Phe-moiety and a glutaric acid spacer and was formed as a new cytotoxic SST-drug conjugate AN-238 **(Figure 4)** [2]. As reported, the conjugate AN-238 could significantly inhibit the growth of various tumors expressing SSTR2a, SSTR3 and SSTR5 [47].

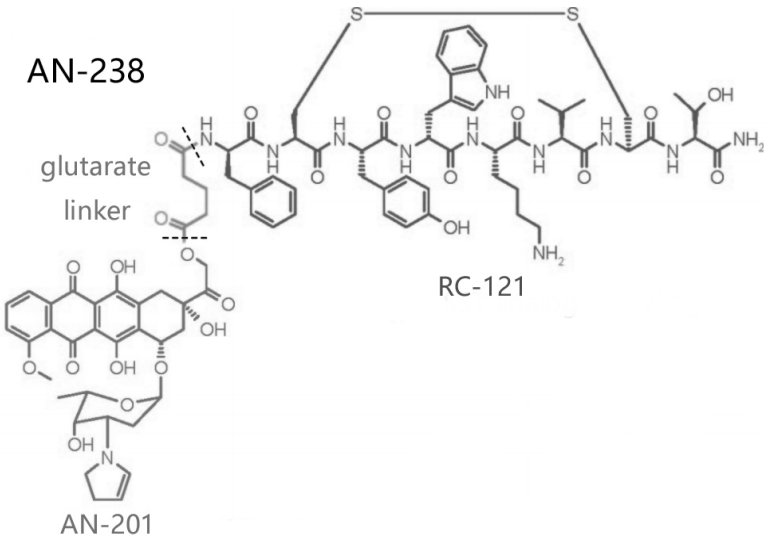

Figure 4. Molecular structure of the cytotoxic SST drug conjugate AN-238, which consists of RC-121 covalently linked to 2-pyrrolino-DOX by a glutaric acid spacer.

This drug conjugate AN-238 has demonstrated to have the anti-proliferative activity in the *in vitro* studies on various human cancer cells, including cells from MDA-MB-231 breast cancer, PC-3 prostate cancer, MIA PaCa-2 pancreatic cancer, and MKN-45 gastric cancer [66]. The effects of AN-238 were also confirmed *in vivo*. AN-238 was investigated in SSTR-positive MCF-7-MIII, MDA-MB-231 and DOX-resistant MX-1 human breast cancer xenografted into nude mice. After 60 days of single intravenous injections of AN-238, the tumor volume continued to be suppressed, whereas AN-201 was ineffective and had severe toxic side effects. AN-238 is also a good drug candidate for the treatment of ovarian cancer. Its corresponding receptor is found in >76% of human ovarian cancer specimens. Thus, the toxicity and anticancer effect was evaluated in UCI-107 human ovarian cancer

cells xenografted into nude mice. The significant inhibition of UCI-107 tumor growth and the tumor volume was decreased by more than 67% after two intravenous injections of AN-238. In a recent study, SSTR2a and SSTR3 were detected in 43% and 39% of the human endometrial cancer specimens, respectively [67]. Accordingly, AN-238 was investigated in HEC-1A, RL-95-2 and AN3CA human endometrial cancers, which were SSTR-positive. Results showed that AN-238 significantly inhibited the tumor growth of these cancers, whereas AN-201 was inactive. A strong inhibition of the growth of SW-839 and 786-0 renal cell cancers xenografted into nude mice was obtained after treatment with a single dose injection of AN-238 [68]. In nude mice bearing H-69 small cell lung cancer (SCLC), AN-238 caused the significant inhibition of tumor growth. Treatment with AN-238 also inhibited the growth of H-838 non-SCLC cells because of the presence of SSTR2 and SSTR5 in tumor vasculature [69]. Furthermore, AN-238 strongly inhibited the growth of Hs746T and NCI-N87 human gastric cancer cells, which expressed a high concentration of SSTR2 and SSTR5. The cytotoxic SST analog AN-238 also reduced the tumor growth of intraosseously implanted C4-2 and subcutaneous MDA-PCa-2b human prostate cancer cells xenografted into nude mice [70]. The SST conjugate AN-238 displayed its broad anti-tumor activities in suppressing the growth of tumors grown from cell carcinoma cells, human glioblastoma cells, pancreatic cancer cells, colorectal cancer cells, and non-Hodgkin's lymphoma (NHL) cells, with the high expression of SST receptor subtypes 1, 2, 3 and 5. AN-238 inhibited the growth of various SSTR-positive tumors and particularly demonstrated indirect antitumor activity against the human SSTR-negative non-SCLC NCL-157 xenografts by directly affecting SSTR-positive tumors in blood vessels of mice [62]. There are some other SSTR2-targeted SST conjugates that also have been reported for their anti-tumor activities [71].

Cytotoxic BN/GRP Drug Conjugates

BN/GRP and its receptors

BN is a 14-aminoacid peptide that was isolated from frog skin. The BN-like peptide GRP consists of 27 amino acid residues, with its carboxyl terminal decapeptide similar to that of BN. Consequently, the two peptides have similar biological activities [63]. Both peptides have several physiological functions as neurotransmitters and gastrointestinal hormones. In addition, these peptides function as growth factors and modulate tumor cell proliferation [72]. Certain cancer cells can synthesize BN and GRP. Cuttitta et al. [73] found that SCLC cells can secrete and respond to BN and GRP. GRP were deemed to play a role in various cancers such as prostate, breast, and pancreatic cancer. Both BN and GRP can mediate their actions via membrane-bound cognate receptors, which have at least four different subtypes [63]. These receptor subtypes are the neuromedin B-preferring subtype (NMB-R or BB1) [74], the GRP-preferring subtype (GRP-R or BB2) [74], the BN receptor subtype 3 (BRS-3 or BB3) [75] and the non-mammalian BN subtype 4 (BRS-4 or BB4) [76]. These BN/GRP receptors have been demonstrated in various human lung, breast, prostate, gastric, and pancreatic cancers and cancer cell lines [77,78]. These characteristics have been applied for receptor-targeted therapeutics via BN/GRP drug conjugates.

Anticancer agents linked to BN/GRP analogs

The chemical preparation of highly active cytotoxic SST hybrids containing DOX or 2-pyrroline-DOX was used for the synthesis of cytotoxic BN-drug conjugate. The cytotoxic BN-drug AN-215 was prepared by linking the N-terminal of the BN antagonist RC-23094 (Gln-Trp-Ala-Val-Gly-His-Leu-ψ(CH2–NH)-Leu-NH2) via a glutaric acid spacer to the 14-OH group of DOX AN-201 [79]. This BN-drug conjugate was determined to target BB1 and was identified for its antitumor efficacy in various tumor models **(Figure 5)**.

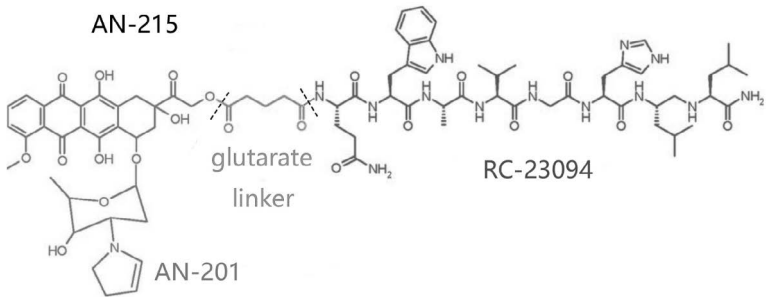

Figure 5. Molecular structure of the cytotoxic BN drug conjugate AN-215, which consists of a BN-like carrier octapeptide RC-23094 that is covalently linked to AN-201 by a glutaric acid spacer.

Antitumor evaluation of cytotoxic BN/GRP drug conjugates

The expression of BN/GRP receptors has been observed in more than 70% ovarian cancers, 33% human breast cancers, and a high percentage of human endometrial cancers [80]. The effect of the cytotoxic BN-drug AN-215 was evaluated in various cancer cells such as the UCI-107, ES-2, and OV-106 human ovarian cancer cells, the MX-1 human breast cancer cells, and the HEC-1A, RL-95-2 and AN3CA human endometrial cancer cells. All assays showed that AN-215 significantly inhibited the growth of these tumors and prolonged the survival of nude mice bearing the tumor xenografts [46]. In another study, the effects of AN-215 were tested in U-87MG human glioblastomas expressing BB1 and BB2. Treatment of nude mice bearing U-87MG human glioblastoma with AN-215 significantly extended the tumor doubling time from 4.5 d to 8.2 d and significantly inhibited tumor growth as demonstrated by a 65% decrease in the final tumor weight and a 70% decrease in the final tumor volume compared with the controls [81]. Furthermore, AN-215 was observed for its inhibition of the growth of tumors from ACHN, 786-0 and A-498

RCC cells xenografted into nude mice, with the inhibitory rates being 59%-68%, whereas the cytotoxic radical AN-201 was inactive [82]. Also, AN-215 significantly inhibited the growth of PC-3 human prostate cancer cells that aberrantly express subtype 1 BN receptors. AN-215 also inhibits the growth of DU-145 and MDA-PCa-2b human prostate cancers. In order to assess the inhibitory effects of AN-215 in SCLC tumors. Male nude mice with the xenografted H-69 SCLC cells intravenously received 200 nmol/kg AN-215 or an equimolar dose of free AN-201. Results showed that AN-215 can powerfully inhibit the growth of H-69 SCLC tumors, whereas AN-201 only produced minor tumor inhibition and severe toxicity [83]. Furthermore, the targeting of AN-215 to the bombesin-binding sites on H-69 SCLC cells in the presence of the bombesin receptor-negative non-SCLC cell line NCI-H-157 was demonstrated *in vitro* by microsatellite markers [2]. Certain other BN/GRP drug conjugates have been reported as well. These drug conjugates may be the potential strategies for the treatment of tumors over-expressing BN/GRP receptors.

CELL-PENETRATING PEPTIDES (CPPS) AS CYTOTOXIC DRUG DELIVERY VEHICLES

Cell-Penetrating Peptides (CPPs)

Cell-penetrating peptides (CPPs) are able to pass through cell membranes. CPPs are also known as protein transduction domains (PTDs). More than 100 different CPPs have been discovered or synthesized in previous decades. CPPs have been grouped into two classes in terms of their functions, sequences, mechanisms of uptake, origin and polarity [84]. The first class has lysine (Lys) as the main contributor and consists of amphipathic helical peptides such as the model amphipathic peptide (MAP) and transportan. The second class is composed of arginine-rich peptides such as Antp, Tat (48-60), oligoarginine and penetratin (pene).

The nuclear transcription activator protein Tat is a CPP and is encoded by HIV type 1 (HIV-1). The CPP Tat is a peptide with 101 amino acid residues that is required for viral replication [85]. Subsequent studies found that Tat (48-60) is the basic domain for cell internalization [19]. Antp is another homeodomain transcription factor, which was first isolated from Drosophila. The basic domain is Antp (43-58), which is also called pene (Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys) [20]. MAP is another peptide consisting of 18 amino acid residues (Lys-Leu-Ala-Leu-Lys-Leu-Ala-Leu-Lys-Ala-Leu-Lys-Ala-Ala-Leu-Lys-Leu-Ala). This peptide was designed by Oehlke et al. [86]. Some of these CPPs have been investigated for the delivery of cytotoxic drugs.

Cytotoxic CPP Conjugates

The conjugation of cytotoxic drug such as DOX to hydrophilic CPPs such as Tat is a widely adopted strategy to improve drug solubility, cell uptake, and anti-tumor potency. The conjugation of DOX to the C-terminal of Tat powerfully inhibited the growth of both drug-sensitive and drug-resistant cervical cancer cells, whereas the conjugation of DOX to the N-terminal showed no significant improvement in cytotoxicity when compared with free DOX [87]. In addition, the conjugation of the anti-tumor agent taxol to the octa(D-arginine) (r8) peptide via disulfide linkers can provide such benefits as improved aqueous solubility, lengthened pharmacokinetics, altered (localized) biodistribution, and most importantly, the ability to overcome the MDR elicited by treatment with taxol alone [88]. Furthermore, peptide r8 also show higher accumulation rates in tumor xenografts than the peptides Tat and pene. The intravenous injection of the r8-DOX conjugate (4 mg/kg) significantly inhibited tumor cell proliferation without significant weight loss, whereas the administration of free DOX (6 mg/kg) yielded similar results, but leading to heavily toxic side effects and significant weight loss [89]. Aroui et al. [90] showed that another DOX conjugate CPP-DOX can effectively induce cell apoptosis in the human breast cancer MDA-MB-231 cells. Furthermore, pene has been demonstrated to be effective as Tat for the efficient delivery of DOX into cells [91]. When conjugated to DOX, both Tat and pene conjugates strongly inhibited the growth of Chinese hamster ovarian cancer cells and MDA-MB-231 drug-resistant cells.

Increasing Cell Specificity of Cytotoxic CPP Conjugates

CPPs as drug delivery vehicles have been demonstrated to improve antitumor efficacy of cyctotoxic drugs to overcome MDR in cancer cells. However, the lack of cell specificity remains the primary drawback of CPP drug conjugates in clinical trials. Several strategies have been established to improve this characteristic. For instance, one alternative strategy of increasing CPP's specificity in tumor cells and tissues is the conjugation of CPPs to cell-targeting peptides or homing peptides [92]. Another one is enhanced permeability and retention (EPR). In addition, hyperthermia-controlled CPP drug delivery and activatable CPPs (ACPPs) are novel approaches that were established in recent years.

CPPs in combination with cell-targeting peptides or homing peptides

Targeted delivery can be performed by cell-targeting peptides or homing peptides with the ability to selectively recognize molecular markers or cell surface receptors in tumor cells or in tumor blood vessels. Besides various known peptides, screening of phage-display libraries has led to the discovery of numerous homing peptides [93]. The cyclic peptide PEGA (CPGPEGAGC) was screened from phage-display libraries and has been shown to accumulate in tumors grown from the breast cancer MDA-MB-231 cells [94]. PEGA in conjugation with the CPP pVEC could enhance the accumulation in different breast cancer cells *in vitro* and *in vivo*. The *in vivo* experiments showed that the homing capacity of these conjugates was highly conserved. These conjugates were found mainly accumulated in tumor vasculature and consequently uptaken by tumor cells [94]. Furthermore, the anti-tumor agent chlorambucil coupled to these conjugates exhibited antitumor efficacy more than four times than that when used alone

[95]. In another study, the peptide RGD (Arg-Gly-Asp) which recognizes integrin receptors in combination with CPPs was shown to home to breast carcinoma and malignant melanoma xenografts [96]. The peptide RGD has high affinity toward integrin αvβ3, which is plentifully expressed in most cancer cells [96,97]. Octa(L-arginine)(R8)-RGD-liposome loaded with paclitaxel (PTX) induced the strongest proliferation inhibition and cell apoptosis against C6 glioma cells. The *in vivo* studies showed that this complex could pass through blood brain barrier (BBB) and efficiently deliver PTX into the brain [98]. Thus, CPPs in combination with cell-targeting peptides could enhance antitumor selectivity.

Enhanced permeability and retention (EPR)

The endothelial lining of the blood vessel wall becomes more permeable in tumors than in normal tissues [99]. Consequently, macromolecular drugs can leave the vascular bed and accumulate in the interstitial fluid. These macromolecules can enter the tumor interstitial space via the high permeability of the tumor vasculature, whereas compromised lymphatic filtration makes the molecules remain in the tumors [100]. Several CPPs have been utilized to enhance the cell membrane penetration of various macromolecules such as liposomes and dendrimers [101]. These macromolecules were loaded with anticancer agents via passive tumor targeting and accumulate in the pathological area, where the conjugated agents were released to exert their anticancer effect. The photostable fluorescent organic dots with aggregation-induced emission (AIE dots), which were modified by Tat, caused the enhanced and prolonged emission efficiency in glioma C6 xenografts [102].

Hyperthermia-controlled CPP delivery

A mild thermal stimulus was developed to control the 'shielding/deshielding' of CPPs and facilitate drug delivery via the temperature. Some temperature-sensitive biopolymers have been incorporated into CPP-conjugated agents such as a thermo-sensitive liposome (TSL) or elastin-like polypeptide (ELP), which enable a phase transition from liquid to solid (or the reverse) depending on the temperature, thereby facilitating the targeted delivery of agents to tumor tissues when used with the focal application of local hyperthermia [103]. TSLs contain lipid materials with a solid-to-liquid phase transition upon heating in a mild-hyperthermia range (40–42°C). In most cases, the CPP-conjugated agents are encapsulated into liposomes and combined with hyperthermia. These conjugates could selectively increase the local drug concentration and obviously improve the anticancer efficacy [104]. Viglianti Benjamin et al. reported that, with each unit of increase in the temperature from 39°C to 42°C during mild hyperthermia, the accumulation of liposomes can accelerate and be doubled [105]. Yang et al. constructed a TSL containing the NGR peptide (Asp-Gly-Arg, which is known as a homing peptide and binds to CD13 subtypes expressed in various tumor vessels). As the targeting moiety and heat-trigger, the CPP-DOX conjugate enhanced specific cancer therapeutic efficacy. This drug delivery system showed good physicochemical properties and biocompatibility. Furthermore, it exhilarated strong tumor inhibitory activities *in vitro* and *in vivo* [106].

The ELP is derived from a structural motif in mammalian elastin and only contains poly(Val-Pro-Gly-Xaa-Gly), where the Xaa is any amino acid except for Pro [107]. The ELPs are temperature-sensitive biopolymers, which undergo inverse temperature phase transition in response to the increased temperature. The polymers can be reversibly transmitted between the liquid and solid phases according to the ambient temperature. Therefore, ELPs are soluble in aqueous solutions at the temperatures below the transition temperature (Tt). Once the temperature exceeds Tt, the ELPs change their conformation and form polymeric aggregates. These phase transition properties can be exploited in combination with local mild-hyperthermia to cause aggregation at tumor tissues. Thermally responsive ELPs combined with hyperthermia exhibit a two-fold increase of their accumulation in heated tumors compared with the same polypeptide without hyperthermia [108].

ELPs combined with CPPs can increase the cellular intake rate. The CPP-ELP combination has been reported to be loaded with a variety of anticancer agents. For example, Massodi et al. [109] reported that, in an *in vitro* model, Antp–ELP showed an uptake rate that was seven times greater than that of ELP alone. Furthermore, Bidwell et al. [109] demonstrated that the combination of Bac (a type of CPPs)-ELP–H1 (c-Myc inhibitory peptide) could target the tumor sites via focused hyperthermia. This polypeptide construct could aggregate at tumor vasculature when combined with focused hyperthermia. The aggregation resulted in a gradient concentration between the vasculature and tumor tissue, thereby facilitating the diffusion of ELPs into the interstitial fluid, while aggregation process can be reversed by removing hyperthermia, accelerating the rate of infiltration of ELP conjugates to the tumor tissue when hyperthermia retreats entering the tumor cells and further inhibiting tumor cell proliferation.

Activatable CPPs (ACPPs)

Most CPPs are not cell specific. To address this challenge, researchers have proposed activatable CPPs (ACPPs) where the cell-penetrating function of CPPs is masked by a polyanionic peptide. Thus, an ACPP consists of a polycationic CPP (an enzyme- or pH-sensitive substrate) and a polyanionic inhibitory domain [110]. This structure is inactive in systemic circulation because the cell-penetrating function of CPPs is efficiently blocked by intramolecular electrostatic interactions with the polyanionic domain. The tumor microenvironment in tumor tissues is different from that in normal tissues. For instances, the concentration of cancer associated proteases (CAPs) is higher in tumor microenvironment, with the pH being lower. The microenvironment can activate the cell-penetrating function of CPPs and then deliver non-specific drugs to tumor cells.

Cancer associated proteases (CAPs) belong to a family of proteolytic enzymes those are often present in tumor tissues at

high concentrations, but are usually down-regulated in normal tissues. CAPs play a key role in tumor invasion and metastasis [111]. CAPs include some cathepsins, urokinase plasminogen activators, and several matrix metalloproteases (MMPs). MMPs are a family of Zn-dependent endopeptidases that cleave the components of the extracellular matrix. This phenomenon is a hallmark of cancer. Furthermore, among MMPs, the levels of MMP2 and MMP9 were found to be highest in various cancers [112]. Peptide sequences that are remarkably sensitive to the cleavage by matrix metalloprotease-2 and -9 (MMP2/9) have been demonstrated, including PLGLAG [113] and PLGCAG [114]. Shi et al. [113] developed an ACPP that could deliver anticancer agents to tumor tissues, with DGGDGGDGGDG as the inhibitory or attenuating sequence, PLGLAG as the MMP2/9 cleavable linker, and r9 as a polycationic CPP. This construct was loaded with DOX and effectively targeted tumor cells rich in MMP-2/9 such as the human fibrosarcoma HT-1080 cells [113].

The tumor microenvironment is known to be weakly acidic (pH 5.7-7.0) in contrast to normal tissues. Thus, some acid-sensitive shielding could be used to block the cell-penetrating function of CPPs. The acid-sensitive ACPP is inactive in the systemic circulation. After entering the tumor interstitial fluid, ACPP's activity can be triggered by the pH, thereby inducing rapid cellular uptake by tumor cells. Cheng et al. [115] reported an ACPP (CR8G3PK6) with a shielding group of 2,3-dimethylmaleic anhydride (DMA), which was conjugated with DOX to construct a novel prodrug (DOX-ACPP-DMA) for tumor-targeted drug delivery. In this hairpin structure, r8 functions as a CPP, whereas G3P is a linker. The shielding group of DMA is linked to the primary amines of K6 via the amide bond. DMA was used to block R8 via intramolecular electrostatic attraction at a physiological pH 7.4. In tumors with an extracellular pH 6.8, the acid-labile amides would be quickly hydrolyzed [116,117], thereby leading to charge reversal [118] and activating the pristine function of CPP with improved cellular uptake of tumor cells.

CONCLUSION

Peptide drug conjugates and some other target strategies have been developed to ensure the efficient and safe delivery of non-specific chemotherapeutic agents, and improve their anti-tumor efficacy while reducing toxic side effects of these agents. These cytotoxic and receptor-specific peptide conjugates exhibited remarkable achievements in targeting tumor cells, suppressing tumor growth and overcoming MDR of tumor cells. They have been demonstrated for their more effective anti-tumor efficacy than conventional non-specific drugs. The evidences support that peptides, with their cognate receptors being highly expressed in cancer cells and able to be applied as drug targets, may be potential alternatives and clinical applications in improving anti-tumor selectivity and overcoming toxic side effects.

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CONFLICT OF INTEREST STATEMENT

There is no any conflict of interest.

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