Published in final edited form as:

Curr Drug Targets. 2018 February 19; 19(4): 380–392. doi:10.2174/1389450118666170302094758.

Improved Protein Toxin Delivery Based on ATTEMPTS Systems

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Abstract

Ribosome-inactivating proteins (RIPs) are widely found in multiple species of plants, bacteria and fungi. As a special family of protein toxins, RIPs can inhibit protein synthesis and induce cell death *via* inactivating ribosome in eukaryotic cells. Thus, RIPs have been applied for anti-tumor therapy in the past two decades. However, because of poor cell permeability, nonselective mode of action for tumor cells, poor pharmacokinetic profiles and immunogenicity, their clinical application has been severely constrained. As an effort to overcome these obstacles, tumor-specific monoclonal antibodies (mAb) have been conjugated to RIPs (forming so called “immunotoxins”) specifically to increase their cytotoxicity and provide tumor targeting. Nevertheless, immunotoxins yet have not fully resolved all the issues and critical challenges still remain, such as immunogenicity and inability to penetrate into the deep site of tumor. Hence, a novel cell-penetrating peptide (CPP)-modified ATTEMPTS system based on combination of CPP-mediated penetration and antibody-mediated tumor targeting, with triggerable drug release function, was developed to achieve effective and safe delivery of protein toxin.

Keywords

Protein toxin; ribosome-inactivating protein; immunotoxin; cell-penetrating peptide (CPP); gelonin; tumor-targeting delivery

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

1. INTRODUCTION

Cancer is one of the greatest threats to human health because of the extremely complicated mechanisms and great difficulty in treatment. Although novel targeted therapeutics (*e.g.*, monoclonal antibodies and small molecule inhibitors) have been successfully applied in clinics [1], many problems still remain unsolved, such as drug resistance. Continual efforts for developing novel agents have been made to satisfy the clinical needs. Protein toxins have attracted much attention in recent two decades.

Ribosome-inactivating proteins (RIPs), originally found in plants and microorganism as defending proteins, possess high anti-tumor activity induced by their ribosome inactivation-related cytotoxicity [2]. RIPs, with N-glycosidase enzymatic activity, are able to depurinate the 28S rRNA of the 60S ribosome subunit, thus inactivating the ribosome and inhibiting protein synthesis, and eventually causing cell death [3]. RIPs thus have been known as potent protein toxins for cancer therapy. Furthermore, RIPs have been reported with capability of inducing cell apoptosis through various mechanisms [4]. Therefore, RIPs have been developed as the potential anti-tumor drugs and have undergone various clinical trials.

ATP-binding cassette transporters (typically, P-glycoproteins, P-gp) are mainly responsible for drug resistance based on drug efflux mechanisms. However, the efflux of P-gp is size-dependent, limited to the small substrates (300–2,000 kDa) [5]. Therefore, the macromolecular protein toxins would be able to circumvent the drug-efflux pumps. Moreover, protein toxins have been demonstrated to be effective for overcoming therapy-resistant cancers [6].

2. CHALLENGES OF RIP-BASED CANCER THERAPY

There are multiple issues limiting the clinical success of RIPs, which are closely related to their molecular structures. The plant RIPs are classified into two groups—the single-chain Type I and double-chain Type II. Type I RIP is single-chained, while Type II RIP possesses a ribosome-inactivating A chain and the lectin-like B chain, linked by a disulfide bond. B chain is functioned as a cell-penetrating motif that binds to the cell surface glycolipids or glycoproteins with beta-1,4-linked galactose residues, facilitating the protein to translocate into cells in a non-discriminated manner [7, 8]. As a result, the non-specific intracellular translocation also yields unwanted side toxicity to normal cells and tissues. In contrast, Type I RIPs, without the cell-translocation B chain, are far less cytotoxic than the type II counterparts because of the poor cell permeability. However, once entering cells, a single RIP molecule is sufficient to kill tumor cells, whereas it requires $\sim 10^5$ molecules of small drugs (*e.g.* doxorubicin) to carry out the same task [9]. Moreover, RIPs have a short half-life *in vivo* due to their relatively small size and the consequent rapid renal clearance [10]. Some even have severe immunogenicity, posing safety concerns [11]. Hence, these disadvantages render poor drug ability for RIPs as anti-cancer drug candidates.

In the recent years, researchers have applied advanced drug delivery technology to improve the drug ability of RIPs. For instance, antibody-mediated targeting delivery and PE-Gylation have been employed to address the abovementioned issues.

In the crusade of developing protein toxin-based tumor-targeting delivery, new approaches have been continuously developed to promote RIPs for clinical use. The application of immunotoxins has been comprehensively discussed in other reviews (*e.g.*, in reference [12]), and here we have given a very brief overview of immunotoxins, but have focused on a new approach named “ATTEMPTS” (antibody targeted, [protamine] triggered, electrically modified pro-drug-type strategy) for protein toxin delivery.

3. IMMUNOTOXIN THERAPY

Antibody-RIP conjugates, termed immunotoxins, are designed to increase the cell membrane-permeation and tumor targeting. Similar to the antibody-drug conjugates (ADC), one or more protein toxin molecules are coupled to a monoclonal antibody (mAb) through chemical bonds. The antibody can specifically recognize and bind with an over-expressed receptor on tumor cell surface, thus facilitating tumor targeting and intracellular translocation (Fig. 1). By disguising the toxin as a misfolded protein to be recognized as a substrate for ER-associated degradation (ERAD), intra-cellular delivery of immunotoxin is achieved after it is re-folded to regain the enzymatic activity [13]. Hence, immunotoxins have been considered as the promising candidates in protein toxin-based anti-cancer therapy and have made some success in clinical trials.

Immunotoxins were initially constructed by conjugating the tumor cell-targeting antibodies to the native ricin toxin, which were also termed as holotoxins. In the cellular studies of the holotoxins, the non-specific binding of ricin B chain to the cell surface glycolipids or glycoproteins with beta-1,4-linked galactose residues was attenuated by exposure to a high level of lactose because of the competitive inhibition [14]. The nonselective distribution and binding mediated by ricin B chain were often responsible for the treatment failure in animal models [15]. To address this problem, B chain was usually removed by a reductant, and A chain then conjugated to mAb by using cross-linkers, so as to improve tumor targeting. Immunotoxins constructed with single-chain toxins (aka hemitoxins, the enzymatically active A chains), have now become the main developing tendency [16].

Moreover, the glycosylation of the naturally occurring toxins could cause macrophage uptake, leading to rapid clearance *in vivo* [13]. However, the recombinant toxins without glycosylation can be readily produced from bacterial expression vectors. In addition, recombinant method provides a powerful tool to produce massive recombinant protein toxins with almost the same bioactivity as their native forms at a very low cost. This method is also allowed to fuse single-chain Fv or Fab fragment to form chimeric toxins [17]. On the other hand, when recombinant immunotoxins are prepared using the bacterial expressing system, the elimination of endotoxin during the purification process is important and remains a challenge because even the minimal residue of endotoxin can cause fatal allergic reaction in human body.

Immunotoxins have been investigated for treating several types of cancers in preclinical and clinical studies, such as lymphoma [18–22], leukemia [23–25], small cell lung cancer (SCLC) [26, 27], breast cancer [28–31], colorectal cancer [32, 33] and other solid cancers [34]. Despite the substantial progress, there are still some problems and challenges of

immunotoxins constraining their clinical application. First, the purification and preparation of immunotoxins are complicated and time-consuming. Second, the antibody molecule is too large to enter the deep site of the tumor tissue because of the steric hindrance effect [35, 36]. The firstly-arrived immunotoxins are inclined to bind onto the tumor vessels and thus create a so-called “binding-site effect” against effective diffusion deep inside the tumor mass [37]. Third, the lack of a mechanism for endosomal escape also compromises the cytotoxicity of immunotoxins [38], owing to low efficiency to transport Golgi and ER, and release into cytosol. Last, there is a risk of the potential immunogenicity caused by either the toxin or the antibody itself [27]. This may require rational design of site-specific conjugation so that the antibody can better block the epitope of a toxin but does not hinder the access of the enzymatic domain of the toxin.

4. CPP-MODIFIED ATTEMPTS SYSTEM FOR TUMOR-TARGETING DELIVERY OF TOXINS

4.1. CPP-based Delivery Strategy

Cell-penetrating peptides (CPPs, aka protein transduction domains, PTD) are a class of short peptides, with rich cationic lysine and arginine. CPPs cannot only translocate into cells but also assist macromolecular cargoes like proteins, DNA, or even nanoparticles to penetrate through the cell membrane and into cytosol [39]. The mechanisms for CPP-mediated intracellular delivery have not been clearly understood yet. A commonly accepted account is that CPP translocates its cargo into cell through either direct transduction or endocytosis pathway [40].

Heparin sulphate (HS) proteoglycan, overexpressed on the surface of various tumor cells, is the major binding receptor that shows highly affinity to CPP due to electrostatic interaction [41]. Therefore, the polyanionic heparin is natural antagonist to CPP; if pretreated with heparin, CPP loses its cell penetration ability [42]. Recently, it has also been demonstrated that CPP can interact with neuropilin-1, expressed on the surface of endothelial and some cancer cells, leading to improved tumor delivery [43].

CPP can facilitate its protein cargoes into cell membrane in a form of either covalent conjugation or non-covalent (*e.g.*, physical interaction) complex [44, 45]. Thus, CPP has been applied as a potent tool for intracellular delivery of therapeutic protein drugs. Low-molecular-weight protamine (LMWP) was identified from the enzymatic digestion products of protamine, and has been widely used for protein delivery [46]. For example, LMWP has been used to conjugate with the cell-impermeable L-asparaginase, and thus successfully deliver the enzyme drug into red blood cells (RBC) to treat acute lymphocytic leukemia [47]. The L-asparaginase-encapsulated RBC can extend the drug half-life and reduce immunogenicity of the protein drug.

LMWP also showed the great potential in protein toxin delivery. It has been used for improving intracellular delivery of gelonin, a type I ribosome-inactivated protein found in the seeds of the Himalayan plant *Gelonium multiflorum*. Gelonin possesses high antitumor activity, but is with poor cell-permeability. By chemical conjugation, the cellular uptake

efficiency of the LMWP-modified gelonin was greatly increased, similar to that of the TAT-gelonin (Fig. 2A). TAT is one of the most commonly used CPP from HIV-1 TAT protein. Accordingly, the anti-tumor activity was also enhanced by hundreds folds compared to the non-modified gelonin [48], displaying potent cell penetration capacity. More importantly, LMWP can mediate effective intratumoral penetration of gelonin, showing substantial distribution in the tissue slice, whereas the non-modified gelonin are hardly observed. The intratumoral penetration is useful for gelonin taking action in the deep site of the tumor (Fig. 2B).

4.2. Concept of ATTEMPTS System

Although CPPs have high affinity to some tumor-overexpressing receptors (*e.g.*, heparin sulphate proteoglycan and neuropilin-1), it is still commonly believed that CPPs lack targeting specificity in most cases. Thereby, on one hand, CPP can enhance intracellular delivery; on the other hand, the tumor targeting efficiency still needs to be further improved. To address this problem, a heparin/protamine regulating system combining with antibody and CPP-mediated delivery, called ATTEMPTS, was developed to achieve the site-specific release of CPP-modified protein drugs [46].

The concept of ATTEMPTS was originally developed for targeting delivery of tissue-specific plasminogen activator (t-PA) to thrombolysis with reduced side effects [49–53]. Later on, ATTEMPTS system was applied for delivery of other proteins [46].

ATTEMPTS system is composed of two parts: a CPP-modified protein drug and a heparin-modified targeting antibody. The two components can self-assemble into a tight complex *via* a noncovalent linkage between CPP and heparin, owing to their strong affinity. Once the complex is formed, the negative-charged heparin would mask the cationic CPP, thus disabling the cell penetration ability and decreasing the non-specific penetration of the protein drugs. The system displays a prodrug-like nature, being pharmacologically inactive in a complexation form. The system *via* intravenous administration could target and accumulate in tumor through antibody-directed delivery as well as EPR (enhanced permeation and retention) effect. With a subsequent injection of the trigger agent protamine, the CPP-modified protein would be released from the complex, because of the stronger competitive binding between anionic heparin and cationic protamine, thereby triggering efficient cell penetration of the protein drugs (Fig. 3) [46, 54]. The basis of this design is laid on that CPP and heparin can form a reversible, non-covalent linkage between a protein drug and antibody. More importantly, their binding disables the cell penetration ability of CPP, rendering the protein drug inactive in extracellular space and thus yielding prodrug-type property. The cell penetration of the CPP-drug can be activated by strategically giving the trigger protamine.

4.3. ATTEMPTS for Protein Toxin-based Therapy

As mentioned above, the protein toxins generally confront drug ability problems, such as poor cell permeation, short half-life, high antigenicity and lack of tumor targeting as well. Therefore, it is a formidable challenge to overcome all the issues with a single-functionalized system. ATTEMPTS offers a potential method to address these issues.

The poorly cell-permeable toxins are modified by CPP, and the CPP-toxins have high cellular uptake efficiency. In order to achieve tumor cell-specific penetration, the CPP-toxins are reversibly bound with heparin-conjugated antibody *via* the strong affinity between CPP and heparin. In the thus-formed complexes, CPP-mediated delivery is disabled, and thereby reduces non-specific cellular uptake. Antibody-directed delivery can enhance tumor-targeting efficiency. Moreover, the attachment of the heparin-antibody creates a steric barrier over the toxin molecule, potentially shielding its epitope from binding with the immune initiators in the blood, and reducing the side effects of antigenicity. Type I RIP toxins are generally less than 30 kDa, rendering them subjected to renal clearance from blood stream. The stable complexation of antibody-heparin/CPP-toxin is nanosized, which would not only circumvent the rapid renal clearance but also gain the additional benefit of passive tumor targeting *via* the EPR effect. Once giving the trigger agent protamine, the CPP-toxin is released and its cell penetration ability reinstalled.

4.4. Anti-CEA Monoclonal Antibody as a Targeting Ligand

Carcinoembryonic antigen, a 200-kDa glycosyl phosphatidyl inositol (GPI) cell surface anchored glycoprotein, is overexpressed in a variety of cancers, such as gastric cancer, pancreatic cancer, lung cancer, breast cancer and colorectal cancer, but nearly not expressed in normal tissues [55, 56]. The plasma level of CEA has been used as a biomarker to monitor colorectal carcinoma treatment progress and identify the recurrence after surgical resection [57].

Because of its specific expression in tumors, CEA has been used as a therapeutic target for cancer therapy. For instance, an anti-CEA mAb T84.66 conjugated to the macro-cyclic chelate (90)Y-DOTA was prepared for radioimmuno-therapy in patients with metastatic CEA-producing malignancies, and the conjugates showed promising results in clinical studies [58].

The anti-CEA has also been applied in protein toxin delivery. Chimeric immunotoxins (toxin fused with anti-CEA scFv) have been investigated for treating various CEA-positive cancer cells, displaying 20–2,000-fold enhanced cytotoxicity compared to the native toxin [32]. In order to enhance the intracellular delivery efficiency of an anti-CEA-contained immunotoxin, a CPP (*i.e.*, Arg₉) was introduced the immunotoxin *via* a recombinant method [59]. Although the anti-CEA immunotoxins have been found effective as anti-tumor agents in cellular studies, there is virtually no *in vivo* investigation available. Lately, a novel anti-CEA-modified ATTEMPTS system has been developed to achieve the targeting delivery [60, 61].

4.5. Anti-CEA mAb-contained ATTEMPTS System for Gelonin Delivery

Gelonin, a type I RIP, was found to have a potent anti-cancer ability through its N-glycosidase activity to inhibit cell ribosome, eventually leading to cell death [62]. However, the poor cell-permeability and tumor targeting have constrained its clinical application. To overcome the limitations, an ATTEMPTS system composed of heparin-modified anti-CEA mAb T84.66 and CPP-conjugated gelonin was developed to achieve the safe targeting

treatment. The heparin-linked T84.66 mAb (T84.66-Hep) and the fusion toxin TAT-gelonin can self-assemble into the complex *via* heparin/TAT affinity [61].

4.5.1. Construction of CPP-toxin—CPP-modified gelonin toxin was prepared using either chemical synthesis or recombinant expression. CPP LMWP was conjugated to recombinant gelonin using Traut's reagent and a heterobifunctional PEG (NHS-PEG₃₅₀₀-PDP) as cross-linkers (Fig. 4) [63]. A disulfide bond was designed, which could be cleaved in the reducing environment inside tumor cells.

Alternatively, CPP-gelonin can be prepared using recombinant method as a chimeric fusion protein. Full sequence of LMWP-gelonin or TAT-gelonin gene was inserted to a pET vector containing a thioredoxin (TRX) tag, a 6×His tag and a TEV protease cleave site (Fig. 5). The fusion proteins were expressed using *E. coli* expression system and purified by Ni-NTA affinity chromatography [63, 64].

Both the LMWP-gelonin and TAT-gelonin displayed significantly increased anti-tumor activity, compared to the native gelonin. The LMWP-gelonin, prepared by either chemical synthesis or recombinant method, can enhance the anti-tumor efficacy in various cancer cell lines, demonstrating the CPP-mediated increased cellular uptake (Fig. 6) [63]. Similarly, TAT-gelonin fusion protein also showed a significantly enhanced cytotoxic activity, compared to gelonin (Table 1) [64].

Furthermore, the prodrug-type function of CPP-toxin was investigated by using heparin as a blocker and protamine as a trigger. The TAT-mediated cell penetration can be inhibited by the polyanionic heparin, forming a heparin-masked TAT-gelonin, but reversibly restored by adding protamine (Fig. 7), providing a proof concept for the ATTEMPTS application.

4.5.2. Preparation of Heparin-linked Antibody—Heparin-linked T84.66 mAb was chemically synthesized using Traut's reagent and a heterobifunctional PEG (MAL-PEG₃₅₀₀-NH₂) as cross-linkers, as illustrated in (Fig. 8) [61]. Carboxyl groups in heparin was activated by (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), and reacted with the amino group of PEG. The thus-formed heparin-PEG-maleimide can then conjugate to the thiolated T84.66 mAb, yielding the T84.66-heparin.

4.5.3. In Vivo Application of CEA-targeting ATTEMPTS System in Colorectal Cancer Therapy—The CEA-overexpressing colon cancer cell line LS174T was used. The blockage of cell penetration was tested by addition of heparin to TAT-gelonin, and the cell proliferation was measured. It was found that the cytotoxicity of TAT-gelonin was diminished after addition of T84.66-Hep, displaying heparin dose-dependent effect (Fig. 9A). The ratio of 1:3 (TAT/heparin) can achieve full inhibition. However, the pharmacologically inactive ATTEMPTS system of T84.66-Hep/TAT-gelonin could be activated by the addition of protamine, and the cytotoxic TAT-gelonin was released and killed the tumor cells. At above 5:1 molar ratio (heparin/protamine), the activity (IC₅₀) was completely restored, similar to that of TAT-Gel alone, demonstrating the success of the heparin/protamine regulating strategy (Fig. 9B–C) [60].

In vivo imaging study was carried out in the CEA-overexpressed LS174T xenografted tumor bearing mice to investigate the targeting delivery and biodistribution of the T84.66-Hep/TAT-gelonin ATTEMPTS system. Compared with TAT-gelonin, the system achieved prolonged circulation and enhanced accumulation at tumor site after tail vein injection via antibody-directed targeting and EPR effect as well (Fig. 10). The fluorescence intensity measurement showed a significantly enhanced tumor exposure (43-fold) at 24 h for the ATTEMPTS system, compared to the TAT-gelonin.

In vivo treatment showed that the ATTEMPTS system had enhanced anti-tumor activity that was triggered by the systematically given protamine, in comparison with other groups (Fig. 11A–B). It indicated that after anti-CEA-directed tumor delivery, protamine effectively triggered the release of TAT-gelonin and its cell transduction. The T84.66-Hep/TAT-gelonin without protamine also had better treatment outcomes than the TAT-gelonin, probably due to the increased drug accumulation in tumor and T84.66 mAb-mediated endocytosis. The trigger agent protamine alone did not show any attribution to the inhibition of tumor growth.

It should be mentioned that despite the beneficial therapeutic effects, potential toxicity (body weight loss) was observed after the protamine administration (Fig. 11C), probably caused by the non-specific activation of prodrug in circulation or normal tissues. Therefore, the timing of protamine administration needs to be further optimized [60].

The work demonstrated the feasibility of the CPP-based ATTEMPTS system for treating CEA-overexpressed colorectal cancer. It offers an effective and safe approach for tumor-targeting toxin delivery.

Of note, tumor-homing peptides (THP) have recently gained great interest of scientists. THP can recognize the specific tumor surface receptors. Hundreds of THPs have been identified, of which the sequences are available in the THP database [65]. THPs have been used in nano-drug carriers to selectively deliver nanoparticles into tumor cells [66]. Hence, THP is an option to replace antibodies in protein toxin-based tumor targeting delivery.

5. SMART PEGYLATED IMMUNOTOXIN DELIVERY

Toxin conjugating with protein ligands is like a miniature of immunotoxin. Non-Ab targeting ligands, such as transferrin (Tf), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and Interleukins (IL), have been investigated for preparation of toxin targeting delivery systems [67–71]. The targeting ligands recognize the receptors over-expressed on tumor cell surface, and thus facilitate the endocytosis of toxins and improve their targeted delivery.

Smart PEGylated techniques have been used for protein toxin delivery in order to overcome the problems such as quick metabolism and rapid elimination from the body, and severe immunogenicity. For example, tumor protease-sensitive PEGylation was used for modification of protein toxins. A designed ankyrin repeat proteins (DARPs), which can recognize the epithelial cell adhesion molecule (EpCAM) and function as a targeting ligand, was fused to the N-terminal of *Pseudomonas* exotoxin A (PE) to construct fusion toxin Ec1-PE [72, 73]. In addition, a tumor protease substrate sequence was included in the N-terminal

of fusion toxin. An unnatural amino acid, azidohomoalanine (Aha), was introduced to replace the N-terminal methionine using auxotrophic *E. coli* strain in methionine-free Aha-supplemented medium. The mutant fusion protein was coupled with DBCO-PEG_{20k} through click chemistry [72]. In another study, two Aha residues were introduced in each Ec1-PE by methionine mutagenesis. PEG coupled with tumor protease substrate peptide with a terminal DBCO was conjugated to the fusion toxin by the same method [73]. Both constructions were designed to target EpCAM-overexpressing cancer cells, and toxins could be released and activated specifically at the tumor site *via* tumor protease-dependent cleavage (Fig. 12). They could effectively prolong the plasma half-life and decrease non-specific toxicity *in vivo*.

6. CONCLUSION

RIPs, as potent antitumor agents, have been widely investigated for clinical application. Despite the high ribosome inactivation ability, RIPs confront many challenges for bench-to-bed development. So far, there is not a protein toxin-based drug approved for market yet, though some have entered clinical trials. How to improve the drug ability, including improvement of tumor-specific delivery and tumor cell uptake, immunogenicity, and short plasma half-life, is the pressing issue that needs to be addressed.

Antibody conjugation provides a potential solution to overcome these problems. Conventionally, the antibody-protein toxin conjugates have been applied, and substantial progress has been made. However, there still are some unsolved problems, for example, the multi-steps synthesis, the activity loss caused by steric hindrance of antibody. Moreover, it is difficult for immunotoxins to penetrate into the deep interior of solid tumors [16] and they may block the subsequent entrance of drug molecules because of the binding-site barrier [36].

For those PEGylated immunotoxin-based smart delivery systems, the immunogenicity of PEG can still be detected and PEG-containing vacuoles in cells are observed with PEGylated biologicals [74]. These drawbacks cannot be neglected in the safety evaluation of PEGylated formulation in clinical studies. To overcome them, the branched PEG is favored. Moreover, there are other alternative polymers emerging for protein conjugation [75–77].

In terms of tumor penetration, small-molecule ligands have advantages over antibodies [35]. However, small ligands yield little benefit for prolonged circulation. Therefore, dual-ligand strategy has been applied for protein toxin delivery. ATTEMPTS system contains two active ligands. Antibody is used for improving tumor targeting, and circulation time, while the sequentially exposed CPP for enhancing intratumoral and intracellular delivery of protein toxins. The shielding and de-shielding render the ligands work in a sequential manner, and avoid their every problem. Further investigation will be focused on the optimization of the administration timing of the triggering agent to ensure yielding optimal treatment benefits.

A single-modified system may not be able to solve the multiple tasks in protein toxin delivery. The multi-functionalized systems containing dual or several functional modules,

such as ATTEMPTS, and other smart systems, would be promising to overcome the difficult challenges.

Acknowledgments

We are thankful for the support from 973 Program, China (2014CB931900, 2013CB932503) and NFSC (81172996, 81373357, 81361140344, 81422048, 81673382), as well as from Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2015R1A6A3A 01020598 & NRF-2015R1C1A1A02036781).

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Fig. 1. Schematic illustration of the recognition and internalization process of a targeted toxin in cancer cell. Antibody specifically binds with the receptors, subsequently triggering endocytosis. After escape from the endosomes, the toxin is transported to Golgi and then reaches the endoplasmic reticulum (ER). Through a mechanism of ER-associated degradation (ERAD), the toxin as a substrate for the ERAD system is delivered to cytosol, thus inactivating the ribosomes *via* N-glycosidase activity [13].

Fig. 2.

A) Uptake studies in CT-26 colon cancer cells with Rhodamine-labeled gelonin and the CPP-modified gelonin (nuclei were stained with DAPI). **B)** Tumor penetration studies in the BALB/c mice with xenografted subcutaneous colon tumor. Ten hours after injection of rhodamine-labeled gelonin or LMWP-gelonin, tumors were isolated and cryosectioned, and photographed by confocal microscopy. Reproduced with permission from [48].

Fig. 3.
Scheme of the CPP-modified ATTEMPTS for enhanced toxin therapy for cancer treatment.

Fig. 4.
Scheme of gelonin-LMWP chemical conjugation *via* a disulfide bond using heterobifunctional PEG as spacer.

Fig. 5. Structure of recombinant toxins. **A)** pET-AT-Gel vector. The pET-TAT-Gel vector contains the full sequence of TAT-gelonin gene and thioredoxin (TRX) gene. **B)** TRX-TAT-Gel, TAT-Gel, and rGel proteins. Reproduced with permission from [61].

Fig. 6. Cytotoxicity of recombinant gelonin (rGel), gelonin-LMWP chemical conjugates (cG-L) and recombinant gelonin-LMWP chimera (rG-L) on (A) CT26, (B) LS174T, (C) 9L and (D) PC-3 cell lines. Reproduced with permission from [63].

Fig. 7.

A) Scheme of heparin/protamine-based regulation of cell penetration. **B)** Cellular uptake of TAT-gelonin conjugate. The cell penetration of TAT-gelonin was inhibited by adding heparin and forming heparin/TAT-gelonin complex, thus displaying prodrug-like property. The system can be activated by adding protamine, triggering release of TAT-gelonin. TAT-gelonin was labeled by Rhodamine, and cells were photographed by confocal microscope. Reproduced with permission from [61].

Fig. 8.
Scheme of chemical synthesis of T84.66-Heparin conjugates.

Fig. 9.

ON-and-OFF modulation of the ATTEMPTS T84.66-Hep/TAT-gelatin. **(A)** Inactivation of T84.66-Hep/TAT-Gel. **(B)** Protamine-triggered antitumor activity of TAT-Gel. **(C)** The protamine addition time on effects of protamine-triggered release. After pretreatment with TAT-Gel/T84.66-Hep, cells at varying time points, were given with protamine. Reproduced with permission from [60].

Fig. 10.

Biodistribution of TAT-Gel (**A, C**) and T84.66-Hep/TAT-Gel (**B, D**). With the antibody

T84.66-mediated delivery, the tumor drug accumulation was significantly increased.

Importantly, the residence time inside the tumor also prolonged and substantial fluorescence was still observed even at 72 h post dose. Reproduced with permission from [60].

Fig. 11. *In vivo* treatment efficacy of the ATTEMPTS system. (A) Tumor volumes and (B) representative mice and tumor images at the experimental endpoint. (C) Relative average body weight change (%) during treatment regimen. Reproduced with permission from [60].

Fig. 12.

Tumor protease-responsive PEGylation of DARPin-PE chimeric toxin (left). It can be activated by tumor protease-dependent cleavage to exert enhanced cytotoxic activity (right). Reproduced with permission from [73].

Table 1

Cytotoxic activity (IC₅₀) of rGel and TAT-Gel. Reproduced with permission from [64].

Cell Line		IC ₅₀ (nM)		
		rGel	TAT-Gel	Fold Difference
Cancer cells	LS174T	5130 ± 802	29.0 ± 15.0	177
	CT26	4190 ± 1120	72.2 ± 26.6	58
	U87 MG	3100 ± 409	46 ± 13.7	67
	9L	4260 ± 856	57.8 ± 26.3	74
	HeLa	4980 ± 920	66.4 ± 31.4	75
	PC-3	3330 ± 761	63.4 ± 25.1	53
Non-cancer cells	MDCK	3580 ± 846	53 ± 16.3	68
	293 HEK	4300 ± 1020	46.7 ± 14.0	92