Peptide and protein drug delivery to and into tumors: challenges and solutions

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The potential of peptide and protein anticancer agents has yet to be realized owing to the many unresolved problems concerning their delivery to the site of a tumor and into tumor cells. However, our understanding of the mechanisms underlying the biological fate and biodistribution of protein and peptide drugs has advanced to the stage where methods that use or influence these mechanisms are now available. There are different approaches that can improve the stability, longevity and targeting of peptides and proteins in the body, such as their modification with various soluble polymers, incorporation into microparticular drug carriers, enhanced permeability and retention effect-based tumor targeting and the use of targeting moieties. Furthermore, new approaches to intracellular drug delivery, including the use of transduction proteins and peptides, are being developed. These advances promise the delivery of a new generation of anticancer drugs.

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▼ Many peptides and proteins possess a biological activity that marks them as potential therapeutics, in particular, anticancer agents. Peptides such as the somatostatin analogs octreotide, lanreotide and vapreotide are now available in the clinic to treat pituitary and gastrointestinal tumors [1]. Peptide inhibitors of angiogenesis, including endostatin, are in different stages of clinical development and show great promise as anticancer drugs [2,3]. In addition, research on depsipeptides, bio-oligomers found in microorganisms and marine invertebrates, has revealed a set of potential anticancer agents [4]. L-asparaginase, an enzyme that inhibits the growth of tumors by breaking down asparagine, an amino acid that some types of tumor cell require in much higher amounts than normal cells do, became a standard tool for the treatment of leukemia [5]. Antibodies against certain cancer-specific ligands can also be considered as protein anticancer drugs. Two FDA-approved examples are trastuzumab and Rituxan®, both developed by Genentech Inc. (http://www.gene.com). Trastuzumab is a humanized antibody raised against HER-2/neu, the extracellular domain of the HER-2 protein expressed in certain types of breast cancer. Trastuzumab induces immune-mediated responses, down-regulates the HER-2 receptor and promotes the production of cell-cycle inhibitors [6]. Furthermore, it is well tolerated by patients. Tumor remission was observed in 60–70% of patients when trastuzumab was used in combination with paclitaxel [7]. Rituxan® is a humanized antibody raised against the B-cell-specific antigen CD20. This antibody is effective against hematologic malignancies [8].

Advances in solid-phase peptide synthesis and recombinant DNA and hybridoma technologies enable unlimited quantities of clinical-grade peptides and proteins to be produced. Furthermore, information from the human genome project, together with advances in proteomics, promises further progress in the identification and development of peptide- and protein-based anticancer drugs.

Delivery and distribution issues for peptide and protein drugs

The use of protein and peptides as therapeutic agents is hampered by their rapid elimination from the circulation through renal filtration, enzymatic degradation, uptake by the reticuloendothelial system (RES) and accumulation in non-targeted organs and tissues (Box 1). Because many anticancer drugs are designed just to kill cancer cells, often in a semi-specific fashion, the distribution of anticancer

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Box 1. Proteins and peptides as therapeutic agents

Challenges of peptide and protein drug delivery
- Fast elimination from the systemic circulation because of renal clearance and enzymatic degradation.
- A danger of developing an immune response.
- Uptake by non-target organs and tissues.
- Inefficient cell entry.

Advantages of peptide and protein-polymer conjugates
- Decreased renal filtration.
- Protection against enzymatic degradation.
- Lowered immunogenicity.
- Selective accumulation in tumors via the enhanced permeability and retention effect.

drugs in healthy organs or tissues is especially undesirable because of the potential for severe side effects. Because of the rapid elimination and widespread distribution of drugs into non-targeted organs and tissues the drug needs to be administered in large quantities, which is often not economical and is sometimes complicated by non-specific toxicity.

Many peptide and protein drugs, as well as antibodies, exert their action extracellularly by receptor interaction. However, many others have their targets inside the cell; the low permeability of cell membranes to macromolecules often represents an additional obstacle for the development of peptide- and protein-based anticancer formulations.

Numerous approaches to overcome rapid elimination and non-specific biodistribution of conventional drugs have been developed and can be adapted for the delivery of anticancer peptides and proteins. The simplest way to compensate for the rapid elimination of biologically active substances is to inject the substance of interest at a controllable rate using an external pump and an iv line. A drug can also be delivered to a target organ by administering it directly into the area of interest. However, both methods require skilled medical personnel and often hospitalize the patient for a prolonged period. In addition, these methods and are not appropriate when a drug is very toxic or the site of the desired administration is difficult to reach or is delocalized.

Various implantable devices ranging from erodible polymeric gels [9] to microfabricated chips [10] have been proposed to overcome the low stability and non-specific biodistribution of potential therapeutic agents. Some of these devices are already used in the clinic or are being tested in clinical trials. However, the majority of implantable devices are rather costly. An additional disadvantage is that their insertion and, if needed, removal, require surgical intervention. Their use is limited to sites of delivery that are large enough to accommodate the implantable device and the use of these devices does not address the problem of intracellular delivery of pharmaceuticals. See Refs [9,11–13] for further information on macroscopic delivery systems, including those based on microchip technology.

Effects of polymer conjugation on peptide and protein longevity in the circulation and on tumor accumulation

Benefits of polymer conjugation

Renal filtration and excretion are mainly responsible for the rapid clearance from the systemic circulation of proteins with molecular weights (MW) of 40 kDa or lower. This issue can be prevented by conjugating the biomolecules with water-soluble polymers, which results in a complex of >40 kDa [14]. For example, the circulation half-life in rabbits of a dextran and soybean trypsin conjugate with a MW of 127 kDa is about ten times longer than that of the 20 kDa native protein [15].

Additional benefits of peptide and protein–polymer conjugation include increased resistance against enzymatic degradation and lowered immunogenicity (Box 1). Even endogenous proteins can be susceptible to protease degradation in the bloodstream and interstitial space or induce an immune response. This is typical of recombinant proteins expressed in *E. coli* because these proteins are often non-glycosylated or improperly folded. Enzymatic degradation and an immune response against a protein result in its rapid elimination from the systemic circulation. In addition, the development of an immune response is potentially dangerous because of the possibility of allergic reactions and anaphylactic shock upon repetitive administrations. The mechanism of protein protection by polymer attachment is similar in both cases. Polymer molecules attached to the protein create steric hindrances, which interfere with binding to the active sites of proteases [14], opsonins and antigen-processing cells [16].

Conjugates with poly(ethylene glycol)

Several polymers have been used for protein stabilization with varying degrees of success [14]. Currently, poly(ethylene glycol) (PEG) is the most widely used polymer for the modification of proteins with therapeutic potential. This polymer is inexpensive, has low toxicity and has been approved for internal applications by drug regulatory

agencies [17,18]. PEG is commercially available in a variety of molecular weights and in chemically activated, ready-for-use forms for covalent attachment to proteins [18].

PEG-modified L-asparaginase (Oncospar®), which was proposed as an anticancer agent in 1984 [19], was developed by Enzon (http://www.enzon.com). This formulation was approved as an orphan drug in the USA for use in lymphoma and leukemia treatments [20]. The PEGylated formulation has an apparent circulation half-life in children of 5.7 days compared with 1.2 days for the native enzyme [5]. Oncospar® does not induce hypersensitivity reactions in patients who have had hypersensitivity reactions to the non-modified enzyme [21]. PEGylated interferon α–2b, which was recently approved in the USA by the FDA for treating chronic hepatitis C [22], also shows potential as an anticancer agent because it can stimulate the immune system. The efficiency of an FDA-approved PEGylated version of interferon α-2b against various tumors is well documented and is the subject of clinical trials [23].

Conjugates with poly(styrene-co-maleic acid anhydride)

In some cases, the circulation time of drugs can be increased by conjugating with polymers that are not large enough to prevent renal clearance themselves, but which can attach themselves, with their conjugated drug, to natural long-circulating blood plasma components, such as serum albumin or lipoproteins. An example of such a polymer is poly(styrene-co-maleic acid anhydride) (SMA) [24]. Conjugation of peptides and proteins with this 1.5 kDa polymer increases the circulation time of anticancer proteins and peptides because the conjugates bind to plasma albumin [25]. As with the conjugation of drugs with high-MW polymers, conjugation with SMA protects proteins from enzymatic degradation and decreases the immunogenicity of the modified proteins [24]. At least one protein-based SMA-modified pharmaceutical, neocarzinostatin–SMA conjugate, is currently approved in Japan for treating hepatoma [26]. Several other anticancer formulations based on anticancer peptides and proteins modified with SMA have shown promising results in preclinical studies and clinical trials [24,25].

Enhanced permeability and retention effect

High-MW (40 kDa or higher) macromolecules with long circulation half-lives, including peptides and proteins conjugated with water-soluble polymers, are capable of accumulating in solid tumors via the enhanced permeability and retention effect (EPR) [24,26]. This effect occurs because of certain characteristics of tumor tissues. The first of these is that tumor vasculature, unlike the vasculature of healthy tissues, is permeable to macromolecules with a MW of 50 kDa or even higher. This allows macromolecules to enter into the interstitial tumor space. Another characteristic is that the lymphatic system, which is responsible for the drainage of macromolecules from normal tissues, has decreased function in the case of many tumors [26]. Because of this, macromolecules that have entered tumor tissues are retained there for a prolonged time. Unlike macromolecules, low-MW, conventional pharmaceutics are not retained in tumors because of their ability to return to the circulation by diffusion [26]. See Refs [24,26] for further details on the mechanism of the EPR effect, and the list of preparations for which this effect has been observed.

Microreservoir delivery systems

In many cases, microreservoir (microparticulate) carriers might represent a valid alternative to soluble polymeric carriers. These types of systems include liposomes, micelles, polymer microparticles, and cell ghosts. The use of such carriers results in a much higher active moiety:carrier ratio compared with 'direct' molecular conjugates. They also provide a higher degree of protection against enzymatic degradation and other destructive factors upon parenteral administration because the carrier wall completely isolates drug molecules from the environment. An additional advantage of these carriers is that a single carrier can deliver multiple drug species. All microparticulates are too large to be lost by renal filtration. The main disadvantage of microreservoir carriers is their tendency to be taken up by the RES cells, primarily in liver and spleen [27].

Liposomes and micelles

Among particulate drug carriers, liposomes (Fig. 1) are the most extensively studied and possess the most suitable characteristics for peptide and protein encapsulation. Liposomes are vesicles formed by concentric spherical phospholipid bilayers encapsulating an aqueous space [28]. These particles are completely biocompatible, biologically inert and cause little toxic or antigenic reactions. Their inner aqueous compartment can be used for encapsulation of peptides and proteins. Many techniques for liposome preparation require only manipulations that are compatible with peptide and protein integrity.

However, as with other microparticulate delivery systems, cells of the RES rapidly eliminate conventional liposomes [27]. To prepare liposomes that are capable of delivering pharmaceutical agents to targets other than the RES, attempts have been made to prolong their circulation lifetime. This was achieved with the development of

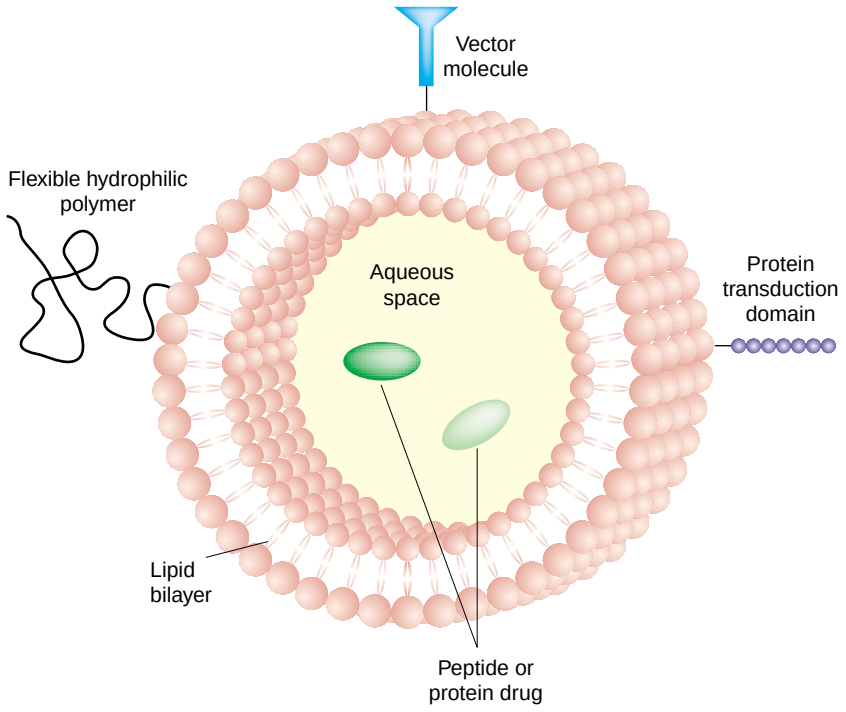

Figure 1. The liposome as a vehicle for protein and peptide drug delivery**.** Flexible hydrophilic polymers [for example, poly(ethylene glycol), PEG] increase circulation half-life by modifying the liposome surface. Tumor targeting of the carrier can be enhanced by attaching vector molecules (e.g. antibodies) to the surface and intracellular delivery can be facilitated by the attachment of protein transduction domains. These peptides target the plasma membrane, allowing the delivery of peptides and proteins in a receptor-independent manner.

surface-modified long-circulating liposomes grafted with a flexible hydrophilic polymer, usually PEG [29]. This prevents plasma protein adsorption to the liposome surface and the subsequent recognition and uptake of liposomes by the RES [30].

Liposomes, in common with macromolecules, can accumulate in tumors of various origins via the EPR effect [31,32]. Currently, liposomal forms of at least two conventional anticancer drugs, daunorubicin [33] and doxorubicin [32], are used in the clinic. Liposomal doxorubicin, incorporated into long-circulating PEG-coated liposomes (Doxil®, ALZA Corporation; http://www.alza.com) demonstrates excellent results in EPR-based tumor therapy and diminishes the toxic side effects of the original drug [32]. Long-circulating liposomes can be easily adapted for delivering peptide and protein-based pharmaceuticals to the tumor.

However, sometimes the liposome size is too large to provide efficient accumulation via the EPR effect, presumably owing to a relatively small vasculature cutoff size in certain tumors [34,35]. In these cases, alternative small-sized delivery systems, such as peptide and protein–polymer conjugates or drug-loaded micelles should be more efficient. Among various pharmaceutical micelles, polymeric micelles, including those prepared from amphiphilic PEG-phospholipid conjugates, are of special interest because of their stability [36]. These particles are smaller than liposomes and lack the internal aqueous space. To load micelles, peptide or protein pharmaceutical agents can be attached to the surface of these particles or incorporated into them via a chemically attached hydrophobic 'anchor'. It has been shown in mice that a model protein can be delivered more efficiently into a tumor with a low vasculature cutoff size using micelles as a carrier than it can be with PEG-liposomes [34].

Tumor cell targeting using specific ligands

Targeting moieties

The use of specific 'vector' molecules that show affinity toward ligands characterisitic for target tissues can further enhance tumor targeting of peptide and protein carriers or make them EPR-effect independent. The use of specific vector molecules is especially important for tumors with immature vasculature, such as tumors in the early stages of their development, and for delocalized tumors. Vector molecules capable of recognizing tumors include antibodies, peptides, lectins, saccharides, hormones and some low molecular weight compounds, such as folate and some vitamins [37]. From this list, antibodies and their fragments have the highest potential specificity and thus provide the most promising opportunity for targeting.

Antibodies as targeting molecules

There are antibodies available that can recognize specific antigens from the majority of known tumors. Among these are antibodies against ovarian cancer, prostate cancer and colorectal cancer [38]. Recent advances in recombinant engineering make it possible to produce anticancer antibodies on an industrial scale at a relatively low cost. Humanized versions of antibodies and their fragments in

which rodent-derived binding sites and human constant regions are combined using recombinant technology are available [39,40].

Among antibodies with anticancer specificity, monoclonal antinuclear autoantibodies (ANAs) with nucleosome-restricted specificity are of particular interest [41,42]. Unlike the majority of anticancer antibodies that recognize only specific tumors, ANAs possess specificity against a variety of tumors. Non-pathogenic ANAs represent a subclass of natural anticancer antibodies, and several of these differentiate between tumor and normal cells [41]. The monoclonal non-pathogenic ANAs 2C5 and 1G3 recognize the surface of numerous tumor, but not normal, cells [41,42]. These ANAs have nucleosome-restricted specificity, and tumor cell surface-bound intact nucleosomes, originating from neighboring apoptotic tumor cells, are their molecular target [42,43]. In addition to their anticancer effects, these antibodies could potentially be used to develop specific delivery systems for a variety of tumor types.

Immunotoxins

A simple targeted delivery system can be constructed by conjugating a vector molecule and a drug moiety. Immunotoxins, molecules assembled from fragments of antibodies and natural protein toxins capable of selective elimination of cancer cells, have shown promising results in clinical trials [44] and represent the best-known example of this approach [45]. Immunotoxins are prepared from natural toxins derived from various sources such as fungi, bacteria and plants. Natural toxins are typically two-chain proteins. One chain is responsible for cell binding and membrane translocation. The other chain is the toxic subunit and usually functions by interfering with protein biosynthesis. Typically, this chain, which is incapable of crossing cell membranes, is non-toxic to cells. The binding chain of natural toxins targets the majority of human cells indiscriminately. Replacing this chain with a cancer-specific antibody or other vector molecules provides a potential tool for the selective elimination of cancer cells [45].

The first generation of immunotoxins suffered from problems typical of other protein-based pharmaceuticals – rapid elimination from the systemic circulation because of enzymatic degradation and an acquired immune response, as well as toxicity to healthy cells. New versions of immunotoxins based on recombinant technology, combining DNA-elements of toxins with binding regions of antibodies, and sometimes growth factors and/or cytokines, are currently under development and show promising results in clinical trials [44].

Targeted microreservoir delivery systems

Vector molecules can also be used for the targeting of microreservoir delivery systems. PEG-modified, long-circulating, doxorubicin-containing immunoliposomes targeted with anti-HER-2/neu monoclonal antibody fragments, represent a recent example of the increased efficiency of targeted delivery systems [46]. In all the studied HER2/neu-overexpressing models, immunoliposomes showed potent anticancer activity superior to that of control, non-targeted liposomes [46]. This superior activity was attributed, in part, to the ability of the immunoliposomes to deliver their load inside the target cells via receptor-mediated endocytosis [46]. This is obviously important if the drug's site of action is intracellular. As with non-targeted liposomes, liposomes targeted by vector molecules can be adapted easily for the delivery of peptide and protein anticancer therapeutics.

Transmembrane delivery

Intracellular targets

The successful delivery of anticancer drugs, including proteins and peptides, into tumors solves only part of a general efficiency problem**.** For many protein and peptide drugs, the next task is to achieve their intracellular delivery, because many targets for anticancer drugs are located inside cells.

In the search for new anticancer drugs, a shift is gradually occurring from an empirical approach based on the evaluation of the efficacy of candidate compounds against cultured cancer cells and animal tumor models to a more rational approach [47]. A huge body of information about cellular, metabolic and signaling pathways essential for tumorogenesis and tumor cell development has led to the identification of protein targets with antitumor potential. Several molecular targets have already been identified [47]. The creation of a working draft of the human genome sequence [48,49], in combination with high-throughput molecular biology methods, promises a continued rapid growth in identifying such targets [50,51].

However, not all protein targets that can be identified and validated by molecular biology tools are considered suitable for drug development, often because of their intracellular location [47]. Myc/Max dimerization, Src homology-2 domain interaction, and Ras/Raf association have been identified as promising targets for the inhibition of tumor development [47,52,53]. Peptide inhibitors of these interactions have been identified but because of the perceived difficulties in peptide drug delivery, companies have concentrated on the development of low MW organic molecules as drug candidates [47]**.** However, these attempts have, to date, been unsuccessful. Peptide inhibitors, which are relatively easy to identify using the phage-display

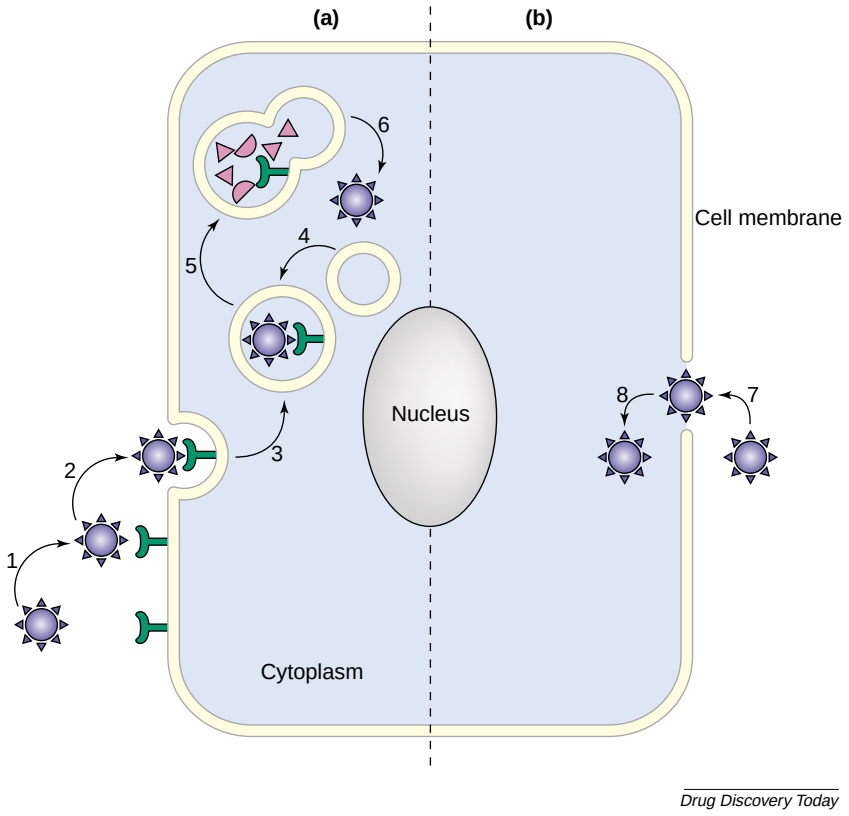

Figure 2. **(a)** Endocytosis versus **(b)** transduction for intracellular drug delivery. Endocytosis: 1. Binding of a drug delivery unit to a specific ligand; 2,3. Formation of endosome; 4. Endosome–lysosome fusion; 5. Degradation of the endosomal content by lysomal enzymes; 6. Possible endosomal escape, and subsequent delivery of drug to the cytoplasm. Transduction: 7,8. Drug delivery units cross the cell membrane and enter the cytoplasm in the intact form and in a receptor-independent fashion.

technique [54] or combinatorial peptide libraries [55], were not themselves considered for drug development, apparently because of their typically (for peptides) poor pharmacokinetics and their inability to reach their molecular targets inside the cells.

Sometimes, tumors result from malfunctions of tumor suppressor genes and the subsequent inactivity of the proteins they encode [51,56]. In this case, the delivery into tumor cells of working copies of recombinant proteins should provide indispensable tools for validating gene function and the potential development of protein- or gene therapy-based methods of treatment. The use of these proteins for molecular target validation and the eventual development of anticancer drugs are again hampered by low permeability across cell membranes.

Receptor-mediated endocytosis

The nature of cell membranes prevents peptide entry unless there is an active transport mechanism, which is usually only the case for short peptides [57]. Vector molecules promote the delivery of associated drug-carriers into the cells via receptor-mediated endocytosis [46]. This process involves attachment of the vector molecule, with its associated drug carrier, to specific ligands on target cell membranes, followed by the energy-dependent formation of endosomes. Cellular uptake via endocytosis is usually efficient, but the delivery of intact peptides and proteins is compromised by insufficient endosomal escape and subsequent lysosomal degradation (Fig. 2). Enhanced endosomal escape can be achieved through the use of, for example, lytic peptides [58–60], pH-sensitive polymers [61] or swellable dendritic polymers [62]. These agents have provided encouraging results in overcoming the limitations of endocytosis-based cytoplasmic delivery, but there is still a need for further improvements or the consideration of alternative delivery strategies.

Transduction

An approach based on the phenomenon of transduction (Fig. 2) is a much more straightforward and efficient way of delivering peptides and proteins to the cytoplasm. It uses the ability of certain peptides to ferry conjugated macromolecules, such as proteins [63] and DNA [64] and even large diameter particles such as 40-nm dextran-coated iron oxide colloidal particles [65,66] and 200-nm liposomes [67,68], across cell membranes directly into the cytoplasm. Peptides that cause transduction (PTDs, protein transduction domains) are derived from proteins of viruses and *Drosophila* Antennapedia transcription factor and can be as short as 10–16mer [63,69,70]. The detailed mechanism of PTD action is not clear, but it appears that these peptides directly target the lipid bilayer of cell membranes [71,72], and penetrate it in a receptor- and/or transporter-independent fashion [63,69]. Several PTD peptides with enhanced protein transduction potential have been synthesized recently [73].

A synthetic peptidyl mimetic containing an N-terminal PTD derived from HIV Tat protein has been shown to inhibit an intracellular protein target, revealing important information regarding the progression of cancer cells

through the cell cycle [74]. In addition, several proteins, including those involved in oncogenesis, cancer-related signal transduction and cell proliferation pathways, have been delivered in an active form into various human cells *in vitro* using fused PTD peptides [75–78]. The Tat PTD has also been used to deliver biologically active proteins into various cells *in vivo* [79]. These results offer new hope for the development of peptide- and protein-based anticancer therapeutics with intracellular molecular targets. The use of PTD-modified liposomes should combine the advantages of microreservoir carriers, enhanced accumulation in tumors via the EPR effect and cytoplasmic drug delivery. Some researchers have highlighted possible fixation artefacts in transduction protein-related experiments [80], but efficient intracellular delivery using these ligands in experimental systems that did not require any fixation provides hope that transduction proteins will have a practical use in drug delivery.

Thus, our current knowledge provides some promising approaches on how to deliver peptide and protein-based anticancer drugs into tumors and tumor cells. New drugs and treatment protocols based on these methods should appear in the near future. More-distant future approaches might also include various methods of gene or DNA delivery into tumor cells, where DNA-based drugs can initiate the biosynthesis of proteins that can serve as anticancer agents or act as vaccines against cancer, using delivery protocols similar to those discussed here.

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