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Issues Related to Targeted Delivery of Proteins and Peptides

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Abstract

While modern genomic and proteomic technology enables rapid screening of novel proteins and peptides as potential drug candidates, design of delivery systems for these biologics remains challenging especially to achieve site-specific pharmacological actions. This article discusses the issues associated with targeted delivery of protein and peptide drugs at physiochemical, physiological, and intracellular levels with a special focus on cancer therapy.

Keywords: targeted delivery, protein therapeutics, peptide therapeutics, cancer therapy

Introduction

Proteins and peptides have long been considered as therapeutic modalities to combat human diseases ever since the commercial introduction of insulin, thyroid hormones, and coagulation Factor VIII in the early and mid-1900s. Mostly produced from natural sources, early application of protein and peptide drugs has been impeded by complicated and costly manufacturing procedures. With advances in recombinant DNA technology and solid-phase synthesis, public interest in protein and peptide therapeutics has greatly increased over the years. Thus far, more than 200 proteins and peptides have received US Food and Drug Administration (FDA) approval for treating a variety of human diseases (www.biopharma.com). While modern genomic and proteomic technology enables rapid screening of novel proteins and peptides as potential drug candidates, design of delivery systems for these biologics remains challenging especially to achieve site-specific pharmacological actions. Proteins and peptides with demonstrated activity on the molecular and/or cellular level often fail to produce sufficient efficacy when applied in vivo, largely because of their unsatisfactory pharmacokinetic profiles. These include: (1) poor oral bioavailability, (2) inadequate stability and shelf life, (3) immunogenicity, (4) short plasma half-life, and (5) poor penetration across biological membranes.

For proteins or protein conjugates of large size, site-specific delivery may be achieved by "passive targeting," ie, the preferential retention of macromolecules in tissues with poorly formed vasculatures (eg, angiogenic tumors and inflamed joints).[1] This phenomenon, known as the EPR (enhanced permeability and retention) effect, is further enhanced by the defective lymphatic drainage normally associated with malignant tissues.[2] At the present time, several proteinaceous anticancer drugs that rely on this passive targeting mechanism have been approved by regulatory agencies, with zinostatin stimalamer (SMANCS) being the prominent example.[3] However, the EPR-mediated passive accumulation of macromolecules does not fulfill the therapeutic potential of protein and peptide drugs at cellular and especially intracellular levels. A more promising approach in this regard is to associate the drugs with ligands or antibodies that bind to receptors or antigens that are overexpressed on target cells, a process termed as "ligand- or antibody-mediated delivery" or in other words "active targeting." With the emerging repertoire of tumor-associated antigens and/or receptors, there is a continuing effort in exploiting biorelevant ligands as the specificity-enhancing moieties to improve the potency and selectivity of therapeutic agents including proteins and peptides. The purpose of this review is to discuss the issues pertaining to antibody- and ligand-mediated delivery systems that target proteins and peptide drugs, with a special focus on cancer therapy.

Classes of Therapeutic Proteins and Peptides

Whether naturally occurring, genetically engineered, or semisynthetic, there is a broad spectrum of protein and peptide drugs, including (1) hormones and growth factors, (2) clotting factors and anticoagulants, (3) bacterial or plant toxins, (4) drug-activating enzymes, and (5) antibody-based drugs. Most (if not all) of the above classes of protein and peptide drugs have been used one way or another in various forms of cancer therapy. It is highly desirable that these therapeutic proteins and peptides possess an "active targeting" capability to reach intended target cells and leave the normal cells unharmed. The "self-homing" hormones

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(LHRH, luteinizing hormone-releasing hormone also known as GRH, gonadotropin-releasing hormone; somatostatin), growth factors (VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; ILs, interleukins), and their agonists or antagonists by and large fall into this category. They have demonstrated some clinical success either by themselves[4] or as delivering ligands (eg, denileukin diftitox[5]). However, it is the monoclonal antibodies that gained the most attention in the past decades as the successful example of targeted protein therapeutics in oncology,[6] either free (rituximab, trastuzumab, cetuximab) or in forms of immunoconjugates (gemtuzumab ozogamicin, ^{131}I-tositumomab, ^{90}Y-ibritumomab).

For proteins and peptides that lack tumor selectivity, coupling to a tumor-specific ligand can significantly modify its pharmacological properties and enhance its tumor specificity (Figure 1). For example, bacterial (diphtheria toxin, *Pseudomonas* exotoxin) and plant (gelonin, ricin) toxins are among the first proteins that have been explored in cancer therapy in a ligand-targeted fashion.[7] To achieve tumor selectivity, protein toxins are structurally altered to remove their normal tissue-binding function before genetically[5,8-11] or biochemically[12-14] linking to a tumor-specific ligand. Although limited by their toxicities, protein toxins are therapeutically beneficial for advanced hematologic malignancies that have become resistant to chemotherapy or radiation.[5,9,15] Prodrug-activating enzymes (eg, carboxypeptidase G2, β-glucuronidase) constitute a unique class of protein drugs that have been selected for site-specific drug delivery.[16] In this approach, enzymes of nonhuman origin are coupled to a tumor-selective antibody,[17,18] a growth factor,[19] or a small molecular ligand (folic acid, carbohydrates).[20,21] After allowing the enzyme conjugate or fusion protein to localize in tumor and clear from the circulation, a specially designed nontoxic substrate (prodrug) is administered and converted to an active drug capable of rapid diffusion into the target tissue (a bystander effect). This 2-step process is designed to improve the efficiency of drug delivery and make the cytotoxic agent more tolerable in humans.[16] Finally, immunomodulators such as cytokines (eg, IL-2,[22-25] GM-CSF [granulocyte-macrophage colony-stimulating factor],[26] TNF (tumor necrosis factor)-α[27]) and costimulatory molecules (B7)[28] have been coupled to antibodies or other ligands directed at tumor cells as a method of activating an immune response on the cell surface. A list of examples of tumor ligand-targeted protein therapeutics is provided in Table 1.

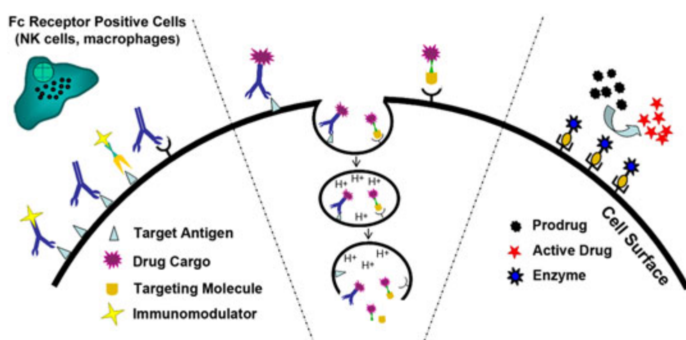

Figure 1. Schematic presentation of targeted delivery of therapeutic proteins and peptides to antigen-positive tumor cells or tumor vasculature. Drugs such as protein toxins are transported to intracellular sites via receptor-mediated endocytosis (shown in the center). On the left, immunomodulators (eg, cytokines) are coupled to a tumor-specific ligand (eg, antibodies, folic acid) and localized on the cell surface to elicit an immune response. On the right, prodrug-activating enzymes are concentrated on the cell surface and can subsequently convert the prodrug into an active drug.

Issues Related to Targeted Delivery of Proteins

Biochemical/Biological Requirements of Protein Drugs

Before a protein drug can be viewed as a candidate for targeted delivery, there are general considerations in regard to its biochemical and biological properties. Unlike small organic drug molecules, proteins often require a complicated higher order of structure (eg, secondary, tertiary, or quaternary) in order to exert their biological functions. Any physical or chemical alterations of the native structure can have a significant impact on a protein's biological activity in vivo. Because of the relatively poor physical stability of proteins, alterations of higher order structure may occur during preparation and storage. Once partially or wholly unfolded, proteins can undergo further changes by aggregation with other protein molecules or form macroscopic ensembles in a precipitation process. The chemical instability may be the result of extra bond formation (eg, disulfide bond formation) and/or cleavage (eg, deamination). While being delivered to the site of action, protein drugs are subjected to harsh physiological factors such as shear pressure in the circulation, proteolysis in the plasma, low pH in endosomes, and digestive enzymes in lysosomes, all of which could lead to deleterious alterations in protein conformation.

The immunogenicity of a protein drug is another major developmental concern because it provokes undesirable host immune responses (ie, the formation of antibodies) against the drug product. Binding of endogenous antibodies to therapeutic proteins accelerates their clearance from the blood, results in loss of efficacy, and precludes repeated administration of the drug. The factors that contribute to such immune incompatibility include (1) proteins of nonhuman origin (eg, bacterial toxins and enzymes), (2) the presence of impurities or aggregates, and (3) the immune status or genetic background of individual patients.[29,30]

Table 1. Examples of Tumor Ligand-Targeted Protein Therapeutics*

Protein Drugs	Targeting Ligands	Disease Indications	References
Diphtheria toxin	IL-2	Cutaneous T-cell lymphoma	5
	Transferrin	Malignant glioma	11
	GM-CSF	Acute myeloid leukemia	15
Pseudomonas exotoxin	anti-CD25	CD25-positive hematologic malignancy	8
	anti-CD22	Hairy cell leukemia	9
	TGF-α	Malignant brain tumors	10
	Folic acid	FR-expressing cancer	12
Momordin	Folic acid	FR-expressing cancer	13
Gelonin	Folic acid	FR-expressing cancer	14
Carboxypeptidase G2 / mustard prodrug	anti-CEA	CEA-expressing cancer	17
β-glucuronidase / doxorubicin prodrug	anti-EpCAM scFv	EpCAM-expressing cancer	18
Carboxypeptidase G2 / CMDA prodrug	VEGF	VEGF-expressing cancer	19
Penicillin V amidase / doxorubicin prodrug	Folic acid	FR-expressing cancer	20
α-rhamnosidase / doxorubicin prodrug	Gal	Hepatocarcinomas	21
IL-2	anti-GD2	Metastatic melanoma	22
	anti-EpCAM	Epithelial ovarian cancer	23
	anti-CD20	CD20-positive lymphoma	24
	MOv19 scFv	FR-expressing cancer	25
GM-CSF	anti-GD2	Neuroblastoma	26
TNF-α	anti-HER-2/neu scFv	HER-2/neu-expressing cancer	27
B7	anti-CD64	Acute myeloid leukemia blasts	28

*IL-2 indicates interleukin 2; GM-CSF, granulocyte-macrophage colony-stimulating factor; TGF-α, transforming growth factor α; FR, folate receptor; CEA, carcinoembryonic antigen; EpCAM, epithelial cell adhesion molecule; VEGF, vascular endothelial growth factor; Gal, galactose alpha 1,3 galactose; GD2, disialoganglioside; TNF-α, tumor necrosis factor α; HER-2, human epidermal growth factor receptor 2.

Although the patient's characteristics cannot be easily changed, the immunogenicity of protein products may be reduced by improving manufacturing process to obtain chemically intact, pure and nonaggregated protein formulations. For antibody-based drugs, humanization of murine monoclonal antibodies is a common practice to avoid human antimouse antibody (HAMA) formation. The immune system can detect even the smallest structural differences in recombinant human proteins. To tackle this problem, Kolkman and Stemmer[31] recommend "exon shuffling" to generate fully humanized DNA sequences without any point mutations that may cause the rise of immunogenicity. When humanization is not an option, the immunogenicity of protein therapeutics may be reduced by introducing minor structural changes (eg, glycosylation, site-specific mutagenesis)[30] or by using polymers (dextran, polyethylene glycol) to shield protein's immunoreactive sites.[32,33] At some point, nonhuman primates and transgenic mice may be used to assess the immunogenicity of newly identified therapeutic protein entities.

The pharmacokinetics of therapeutic proteins is primarily dictated by their size, surface chemistry (eg, charge, glycosylation), and epitope shielding (eg, pegylation). In the body, immediately following intravenous injections, macromolecules are largely confined to intravascular spaces by the continuous capillaries found in most organs except for the kidney and the liver. This has to do with the unique structure of capillary endothelia found in these 2 excretion organs. In the kidney, glomerular capillaries are featured by the existence of fenestrae, circular openings with radii of 20 to 30 nm.[34] Small proteins of less than 70 kDa (especially those with positive charges) can move readily through the renal capillary wall, enter the urine, and cause irreversible clearance from the systemic circulation. For larger proteins that have avoided renal clearance, liver uptake plays a significant role in determining their circulation half-lives. The

capillaries in the liver are more porous with openings of 100 to 10 000 nm, which allows free diffusion of both small and large protein molecules. Following extravastion into the interstitial space, proteins may be retained within the liver by hepatocytes and endothelial cells via electrostatic[35] and/or ligand-receptor interactions (eg, asialoglycoprotein receptor[36]). Residential macrophages in the liver, usually referred to as Kupffer cells, also avidly interact and uptake large protein molecules by phagocytosis. These and other unidentified mechanisms have contributed to the short duration of pharmaceutical proteins. It is important to point out that polymer conjugation (eg, pegylation) effectively alters the properties of a protein drug (size, charge, glycosylation, and immunogenicity), reduces its uptake by the reticuloendothelial system (eg, liver and spleen), prolongs protein circulation, and improves the overall pharmacokinetics and therapeutic index.[33]

Choices of Targeting Ligands and Tumor Specificity

Although highly dependent on the specific nature of the therapeutic "cargo," the choice of ligand for tumor-selective delivery of protein drugs follows a few general principles. First and foremost, an ideal ligand is the one that recognizes a target molecule that is expressed almost exclusively on tumor cells. Since most tumor-associated antigens exhibit low to moderate expression in normal tissues, the relative abundance of target antigen between normal and malignant tissues strongly dictates the selectivity of a targeted drug delivery. However, there have been situations where tumor antigens (eg, PSMA, prostate specific membrane antigen) are localized in normal tissues in such a way that they are inaccessible to drugs in the circulation. In that case, even moderate levels of expression in the tumor may confer good selectivity. For example, PSMA is a molecular marker that has been validated as a promising target for prostate cancer.[37] When compared with expression in normal tissues, PSMA is at least 10-fold overexpressed in malignant prostate and the level of PSMA expression is further up-regulated as the disease progresses into metastatic phases.[38] Since a relatively high level of PSMA has been detected in the brain, some concern has been raised regarding unwanted toxicity to the brain as the result of PSMA-mediated drug delivery.[39] Fortunately in this regard, macromolecules targeted to PSMA are unlikely to have access to the PSMA antigen in the brain owing to the blockade provided by the blood-brain barrier. Folate receptor (FR) is another good example. Access to FRs expressed in a few normal tissues (kidney proximal tubules, choroid plexus) is highly limited by its location on the apical (lumenally facing) membrane of polarized epithelia.[40]

As discussed in this review, tumor-targeting ligands may be divided into 3 classes according to size: small molecular ligands (vitamins, carbohydrates), peptides (bombesin, somatostatin, LHRH, EGF, VEGF), and protein or macromolecular ligands (IL-2, GM-CSF, TNF-α, transferrinn, immunoglobulins). For a selected tumor-associated antigen or receptor, there are usually limited choices of ligands available besides monoclonal antibodies and their bioengineered fragments. Modern antibody technology has allowed the design and preparation of antibodies (whole, F(ab')2, Fab', Fv, scFv) against almost any tumor antigens, whereas high-affinity low molecular ligands are typically in short supply. Given the challenging task of carrying a protein cago, it is prudent to consider the pros and cons of antibodies and smaller molecular ligands in regard to their abilities to achieve tumor selectivity.

Monoclonal antibodies can be made of high specificity and a well-defined affinity (or avidity) to their target. In some cases, antibodies can have direct cytotoxic effects on cancer cells if the antigen it recognizes is a signal-transducing molecule crucial to cell growth or differentiation (eg, anti-HER2/neu, anti-EGF receptor-1).[6] Intact antibodies are immmunologically active in that they are capable of activating an immune response against tumor through interaction with Fc receptors expressed on immune cells (eg, natural killer cells, macrophages, dendritic cells). Variants of antibodies can be made to reduce their immunogenicity, size, and unwanted interaction with normal tissues. In the clinic, monoclonal antibodies and their radioactive or chemotherapeutic analogs have been successful especially in the treatment of hematological malignancies).[6] Nonetheless, antibody-mediated delivery of protein drugs endures several disadvantages in comparison to smaller molecular ligands. These include (1) large size (especially with another protein as a cargo) thus poor penetration into solid tumors, (2) host immune responses still elicited by antibodies that are not 100% human, and (3) diminution of biological activity of the protein drug owing to steric hindrance by the attached antibody. Last, the production and characterization of antibody conjugates or fusion proteins are more complex than preparing each protein agent alone. It is time-consuming and expensive and suffers from all the stability issues associated with a large protein drug (see previous section). In some cases, protein (and peptide) drugs may be entrapped within large macromolecular carriers (liposomes, micelles, nanoparticles) equipped with a tumor-targeting ligand.[41] Such formulation protects the integrity of the protein drug but it may also hamper the ligand's targetability and subsequent release of the protein from the carrier.

Because of their smaller size relative to proteins and macromolecules, peptides and their analogs have become increasingly popular as tumor delivery ligands for imaging agents (eg, ^{90}Y, ^{111}In, ^{99m}Tc, fluorescent dye)[42] and chemotherapeutic drugs (eg, camptothecin, 2-pyrrolinodoxorubicin).[43] Peptides are also biologically active toward tumor vasculature

and/or tumor cells offering a great diversity as an effector and as a "tumor-homing" ligand (discussed in great details in Section 4). Unlike their protein or peptide counterparts, small molecule ligands such as vitamins (folic acid, vitamin B_{12}) and carbohydrates (Gal) have no immediate impact on the mortality of cancer cells upon binding to their targets. They can have nanomolar or subnanomolar receptor binding affinity (eg, folate receptor) and are pharmacokinetically more effective as a protein delivery ligand, because of the absence of immunogenicty (on their part) and the size dependently better tumor penetration. Small molecule ligands such as folic acid are often chemically stable and inexpensive. Additionally, small molecule ligands may allow the preparation of multivalent ligand-protein conjugates. Using folate targeting as a case study, we will discuss, in a later section, issues and potential solutions in the delivery of protein and peptide drugs into cancer cells both in vitro and in vivo.

Tumor Localization of Protein Drugs and Binding Site Barrier

The success of a targeted cancer therapy depends on the ability of the targeted drug molecules to reach primary and/or metastatic sites and to penetrate deeply into the tumor, ie, being available for all binding sites. The tumor vasculature is responsible for the distribution of blood-born drugs into the tumor tissue while tumor penetration (by the ligand-drug conjugates) is largely dependent on a phenomenon called "binding site barrier." As mentioned earlier, tumor vasculature is characterized by leaky and dilated blood vessels with no regular pattern of interconnection.[2] This aberrant vasculature leads to an increased interstitial pressure, hypoxia, and a low pH inside the tumor mass, all of which could affect the delivery of proteins and peptide drugs. Since tumor vasculature is also unique for its expression of biomarkers (eg, VEGFR, vascular endothelial growth factor receptor; alpha(V) integrins) specific for angiogenic blood vessels, many targeted cancer therapies have been focusing on inhibiting vessel formation, thus depriving cancer cells of their nutrients. Still, antiangiogenic therapies alone are not sufficient to promote long-term survival in cancer patients and in many cases they are combined with chemotherapeutic drugs.[44] Unfortunately, as Ma et al[45] reported recently, treating tumor-bearing rats with an angiogenesis inhibitor (TNP-470) followed by temozolomide (TMZ) resulted in decreased tumor uptake of the chemotherapeutic agent, which was associated with the reduction of tumor microvessel density. That is, by destroying the tumor vasculature, one could also damage the "delivering pathways" for subsequent therapeutic agents if multiple treatments are deemed necessary to achieve long-term benefits. To circumvent this problem, Jain[46] and his group have proposed a novel intervention using antiangiogenic agents to "normalize" tumor vasculature instead of destroying them. This is based on the fact that in normal tissues there is a controlled balance between angiogenic stimulators and angiogenic inhibitors whereas in cancer tissues this balance is obviously destroyed. In his studies, Jain[47] used antiangiogenic drugs at a lower dose and with an administration schedule designed to "normalize" rather than destroy the blood vessels. So far, this "normalization" procedure has improved tumor perfusion, decreased interstitial pressure, and resulted in more efficient delivery of drugs into solid tumors. Even after the tumor vasculature is "normalized," a therapeutic drug will face the next challenge of passing through the endothelial barrier to reach tumor tissue itself. For that, proinflammatory cytokines (TNF-α, IL-2, and IL-1β) and vasoactive agents (histamine, bradykinin, leukotriene B4) have been used to improve the vascular permeability.[48] However, systemic administration of these agents are not clinically favorable since the dose required to achieve vascular permeability is typically 10 to 50 times higher than the maximum tolerated dose.[48] On the other hand, targeting cytokines or vasoactive agents with ligands to the tumor vasculature can significantly decrease the overall toxicity, improve tumor uptake, and oftentimes serve a dual purpose of being both vasoactive and cytotoxic.[49,50]

According to the "binding site barrier" theory introduced by Fujimori et al[51] in the late 1980s, ligands with very high affinity for their targets will bind extremely tightly to the first encountered binding sites immediately adjacent to the blood vessel. This creates a physical barrier for subsequent drugs and causes incomplete drug penetration. The effect of binding site barrier also depends on the density of targeted molecules on cell surface (ie, the higher the density, the greater the barrier) and the turnover rate of target molecules.[52,53] Obviously, the binding site barrier is a serious concern for high-affinity monoclonal antibodies and is especially important when antibodies are elected as tumor targeting ligands for protein delivery.[53,54] Although specificity may be sacrificed, 3 approaches may be used to boost the efficiency of tumor penetration, viz, increasing dose, lowering affinity, and decreasing size of a ligand or a ligand-drug conjugate. In guinea pig micrometastases model, Juweid et al[52] showed that increasing the dose of a monoclonal antibody from 30 to 1000 μg only partially overcome the binding site barrier.[55] Adams et al[53] then constructed a series of scFv mutants specific for the HER-2/neu protooncogene with different affinities (10^{-7} to 10^{-11} M). By histochemical and immunofluorescence analyses they found that a lower affinity scFv antibody (3.2×10^{-7} M) diffused broadly into the established human xenografts, whereas the highest affinity scFv antibody (1.5×10^{-11} M) did not traverse more than 2 to 3 cell diameters. Furthermore, biodistribution studies demonstrated that the scFv ligands needed to have a

minimum affinity of $>10^{-7}$ M to achieve any meaningful tumor uptake. The tumor uptake reached a plateau at affinities of $\geq 10^{-9}$ M and eventually decreased at 10^{-11} M.

Whether we choose a small or large targeting ligand, tumor localization and tissue penetration will always be an issue when the actual therapeutic effector is a protein. To guide the design of targeted macromolecular agents, researchers have developed living or artificial tumor spheroids to simulate a 3-dimensional tumor microenvironment and predict the in vivo behavior of these compounds.[56,57]

Cellular Uptake and Intracellular Trafficking of Protein Drugs

As ligand-protein conjugates make their way to the surface of target cells, the plasma membrane constitutes the first substantial hurdle for cellular uptake of protein therapeutics. Besides prodrug-activating enzymes and immunotherapeutic agents, most protein drugs require efficient intracellular delivery to exert their cytotoxic effects. The intracellular organization of mammalian cells is highly complex with extensive compartmentalization that allows the cell to conduct its biochemical processes in a highly regulated manner. These specialized compartments impose additional barriers for protein drugs that need to reach their targets at intracellular loci such as the cytosol and nucleus. Receptor-mediated endocytosis is the primary mechanism for protein entry into the cells upon linking to a tumor-specific ligand. The precise endocytic mechanisms are debatable but may include clathrin-mediated endocytosis, caveolin-mediated endocytosis, macropinocytosis, and even phagocytosis.[58] However, the intracellular destination of ligands (and their protein cargo) is determined primarily by the nature of the receptor/antigen they bind to, not by the way they enter the cell.

In general, after a ligand-protein conjugate binds to a specific receptor on the cell surface, the resulting complex invaginates with its adjoining plasma membrane and develops into endocytic vesicles as they pinch off from the plasma membrane. Following internalization, the newly formed endocytic vesicles quickly become acidified (pH 4.5 to 5.0) and mature into what is known as sorting endosome or early endosomes. The drop in pH within these endosomes promotes the dissociation of ligand-protein conjugates from their receptors. Once released into the luminal side of sorting endosomes, ligands along with their protein cargos are subsequently "sorted" into late endosomal/lysosomal compartments for degradation. The vacated receptors are brought back to the cell surface by distinct recycling endosomes (pH 6.0 to 6.5) for next rounds of transport. Accordingly, the entire endocytic pathway functions as a molecular "Trojan horse" to import protein therapeutics into the cells so long as the protein is coupled to a ligand and the protein cargo does not impair the binding function of the ligand to its receptor.[59]

Strategies have been sought to export proteins (and peptides) prior to their lysosomal degradation or recycling back to the cell surface. For this purpose, ligand-drug conjugates can be made with a cleavable linker to allow the release of free drugs in certain biological milieus (ie, low pH, proteolysis, and reducing conditions).[60] However, for drug molecules to reach their intended site of action, arrangements must be made to ensure their exit from endosomal or lysosomal compartments. The utility of membrane disruption agents has shown promise to enhance endosomal release by undergoing conformational changes in response to the pH gradient.[61] For example, fusogenic peptides are normally unstructured at physiological pH because of self-repulsion of negatively charged residues (glutamate, aspartate).[62,63] Following cellular entry into acidic endosomes, these acidic residues become protonated and form alpha helixes that destabilize the membrane and allow the release of the endosomal content. Using immunoliposomes carrying diphtheria toxin A chain, Mastrobattista et al[62] observed a significantly enhanced cytotoxicity against target cells upon co-encapsulation with a dimeric fusogenic peptide (diINF-7) derived from the influenza virus hemagglutinin. When a synthetic fusogenic peptide (GALA, ie, WEAALAEA-LAEALAEHLAEALAEALEALAA) was anchored on the surface of transferrin-conjugated liposomes, a similar effect was seen with efficient release of encapsulated dye into the cytosol.[63] Since viral-based fusogenic peptides are immunogenic, others have used pH-sensitive polymer (eg, poly[2-alkylacrylic acid]) as carriers for macromolecular drugs to promote intracellular release.[61] While proofs of concept have been established, these pH-sensitive membrane disruption agents are not suitable for the delivery of plain carrier-free ligand-protein conjugates unless the ligand itself can be made to undergo conformational changes in response to low pH.

Photochemical internalization (PCI) is a 2-step technology developed by Berg and others[64] for site-specific intracellular delivery of therapeutic compounds. The mechanism of PCI-mediated endosomal release is relatively straightforward with the use of amphiphilic photosensitizers such as AlPcS2a. These photosensitizers can insert themselves into the plasma membrane and be localized inside the endocytic compartments. When AlPcS2a-loaded cells or tissues are exposed to light, short-lived reactive oxygen species are formed and cause disruption of endosomal and/or lysosomal membrane and the release of all contents from endocytic vesicles. Thus far, PCI has been successfully applied both in vitro and in vivo to protein drugs that lack plasma membrane permeability and are therefore unable to reach their intracellular target. One good example is the plant toxin

gelonin, a type I ribosome-inactivating protein with no inherent cellular binding or translocation domain. The PCI-enhanced intracellular delivery of gelonin was confirmed in a panel of cancer cell lines and in one occasion, greater than 300-fold reduction in protein synthesis has been documented compared with 1.5- to 3-fold reduction with either gelonin or the photosensitizer alone.[64] In a human xenograft model, 67% of mice treated with $AlPcS_{2a}$ (intraperitoneal injection) and gelonin (intratumor injection) were cured after local illumination of the tumor area, as opposed to 10% and none in animals receiving $AlPcS_{2a}$ or gelonin alone (respectively).[65] When applied with gelonin-derived immunotoxin in vitro, a light-dependent synergistic cytotoxic effect has also been reported when the target cells were co-incubated with immunotoxin and a photosensitizer.[66] Obviously, it would be interesting to learn the impact of photosensitizer activation on a variety of ligand-targeted cancer therapies that require intracellular delivery, especially, to establish direct in vivo evidence with systemically administered ligand-drug conjugates. As pointed out by Hogset et al,[67] the potential application of PCI may be limited by poor light penetration into live tissues, restricted local application, toxicity to surrounding tissues, and photochemical damage of the drug itself (which may be resolved by giving light prior to drug treatment).

ISSUES UNIQUE TO TARGETED DELIVERY OF PEPTIDES

Bioactive peptides of fewer than 50 amino acids represent a rich class of pharmaceuticals that are good candidates for the treatment of cancer. Mostly identified through phage display or peptidomimetic libraries, anticancer peptides are designed to interact with specific proteins within tumor microenvironment. Among the biological compounds tested preclinically and clinically, peptides seem to be advantageous because they are small, less immunogenic, and can be easily modified to avoid degradation and improve bioavailability.[68,69] There are several classes of peptides with various modes of action directed against the tumor vasculature or the tumor cell (Tables 2 and 3). Tumor vasculature is unique in that it expresses angiogenic markers that are absent or almost undetectable in established normal blood vessels.[2] Because of their distribution inside the blood vessels, angiogenic proteins are more easily accessible than tumor antigens to peptide drugs in the circulation. Regardless of the situation, tumor-homing peptides can function both as a therapeutic agent (Table 2) and as a delivery ligand for other drug entities (Table 3).

As shown in Table 2A, peptides targeting tumor vasculature are antagonists or inhibitors of angiogenic proteins that include VEGFR,[70] CD36,[71] integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$,[72,73] aminopeptidase N,[74] and matrix metalloproteinases (MMPs).[75] For instance, ATWLPPR peptide is a potent antagonist of VEGF[70]; thrombospondin-1 (TSP-1) induces apoptosis in endothelial cells[71]; RGD-motif mimics block integrin receptors[72,73]; NGR-containing peptides inhibit aminopeptidase N[74]; and cyclic peptides containing the sequence of HWGF selectively inhibit MMP-2 and MMP-9.[75] Laakkonen et al recently identified a LyP-1 peptide which specifically binds to tumor lymphatic vessels and induces apoptosis of endothelial cells.[76] As targeting ligands for tumor vasculature (Table 3A), peptides containing NGR and RGD sequence motifs have been coupled to TNF-α,[81] doxorubicin,[82] tachyplesin (an antimicrobial peptide),[83] and a pro-apoptotic peptide $(KLAKLAK)_2$.[84] When tested in tumor-bearing mice, these peptide-drug conjugates proved to be less toxic and more efficient in eradicating or decreasing tumor burden compared with free peptides or the drugs themselves.[82-84,92]

Since the mid-1980s, researchers have envisioned a class of peptide analogs that block the binding of peptide hormones to receptors expressed in selected human cancers.[93] The most prevalent hormones receptors in this regard[94] are (1) somatostatin receptors[77]; (2) bombesin/gastrin-releasing peptide (GRP) receptor[78]; and (3) LHRH receptor[80] (Table 2B). Since natural peptide hormones are quickly destroyed by plasma proteases in vivo, more stable peptide analogs have to be made for diagnostic and therapeutic purposes. Accordingly, somatostatin analogs were found to have increased in vivo stability while maintaining a moderate to high affinity to somatostatin receptors.[77] In the clinic, these somatostatin analogs (eg, octreotide, vapreotide, lanreotide) have produced modest results in the treatment of human carcinoid, pancreatic, pituitary, and breast tumors.[77] The likely reason for this limited effect is because of the body's rapid adaptation and down-regulation of the subtype 2 receptor, a preferred target for these analogs. Likewise, bombesin/GRP antagonists (eg, RC-3940-II) were shown to have some moderate preclinical activity against various human cancer xenografts.[78,79] Over a 30-year period, Schally et al[80] generated a large array of LHRH agonists and antagonists that are 50 to 100 times more potent than the LHRH itself. In ~70% advanced cases of prostate cancer, LHRH receptor-targeting peptides appeared to be efficacious that they have become the preferred method of treatment. Unfortunately, most of the responses (stable disease or partial remission) were short-lived and most patients relapsed from androgen-independent prostate cancer.

Researchers have developed various radioactive or cytotoxic analogs of hormonal peptides in an effort to improve their efficacy (Table 3B). In 1994, the FDA approved ^{111}In-DTPA-octreotide as an imaging agent for neuroendocrine tumors.[85] By linking therapeutic radionuclide (^{90}Y and ^{177}Lu) to somatostatin analogs, up to 24% complete response and partial remissions were observed in clinical trials of

Table 2. Examples of Tumor-Homing Peptides*

Tumor Targets	Peptides	Modes of Action	References
A. Targeting tumor vasculature			
VEGFR	ATWLPPR	VEGF antagonist	70
CD36	Thrombospondin (TSP)-1 mimetics	Apoptosis	71
Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$	ACDCRGDCFCG (cyclic), SCH 221153 (RGD peptidomimetic)	Ligand mimics	72,73
Aminopeptidase N	CNGRC (cyclic)	Inhibitor	74
MMP-2 and MMP-9	CTTHWGFTLC	Inhibitor	75
Lymphatic vessels	CGNKRTRGC (LyP-1)	Apoptosis	76
B. Targeting cell-surface hormone receptors			
Somatostatin receptors	Octreotide, Vapreotide, Lanreotide	Somatostatin analogs	77
Bombesin/GRP receptor	RC-3940 series	Antagonists	78,79
LHRH receptor	Decapeptyl, Lupron, Zoladex, Cetrorelix	LHRH agonists	80

*VEGFR indicates vascular endothelial growth factor receptor; MMP, matrix metalloproteinases; GRP, gastrin-releasing peptide; LHRH, luteinizing hormone-releasing hormone.

neuroendocrine tumors.[85] In addition, ^{99m}Tc-labeled bombesin[86] and ^{99m}Tc-GRP[87] have been tested in the clinic as tumor-imaging agents. Schally and Nagy[89] synthesized analogs of somatostatin, bombesin, and LHRH carrying doxorubicin or its superactive derivative 2-pyrrolino-doxorubicin. Leuschner et al[90,91] also linked hecate, a membrane lytic peptide to LHRH, fragments of luteinizing hormone (LH), or human chorionic gonadotropin (hCG). Although clinical results are still pending, these compounds were found to be effective in human xenografts of prostate cancer, breast cancer, epithelial ovarian cancer, and brain tumors.[88,90,91] Compared with other tumor-associated antigens, however, hormone receptors are more broadly distributed in the body so that the potential for damage to normal tissues may prevent the use of these cytotoxic hormonal peptides.

An unusual class of peptides consists of naturally occurring or synthetic antimitotic peptides (eg, cryptophycins, dolastatin 10) and cyclic depsipeptides (eg, didemnin B, FR901228, kahalalide F) that are inhibitors of microtubule polymerization more potent than many common chemotherapeutic agents.[95] Often seen with picomolar to low nanomolar IC_{50} in vitro and potent activities in vivo against xenografts, these cytotoxic peptides were disappointing in the clinic owing to their low therapeutic indices.[96,97] As shown in Table 4, to enable the use of these natural toxins, researchers in the pharmaceutical industry have created various antibody conjugates of the synthetic dolastatin 10 analogs, auristatin E and monomethyl auristatin E, targeting CD20,[98] CD30,[99,100] Lewis Y,[99] E-selectin,[101] and TMEFF2[102] tumor antigens. They found that a cathepsin B-sensitive dipeptide (valine-citrulline) linker was superior to an acid-labile hydrozone linker, both of which were designed to facilitate the intracellular release of the active drug.[99] Antibody conjugates with the protease-cleavable linkage were more stable in the plasma and generate therapeutic indices as high as 60-fold in animals with established antigen-expressing tumor xenografts.[99,100] However, these cytotoxic peptides become a part of immunoconjugates that will share the same issues as we have discussed earlier for targeting macromolecules to solid tumors.

Evaluation of Folate-targeted Therapeutic Proteins

Folic acid (pteroylglutamic acid, Vitamin B9) represents a useful ligand for targeted cancer therapy because it binds to a tumor-associated antigen known as the folate receptor (FR). The attractiveness of FR as a tumor target and folic acid as the matching ligand is further enhanced by their high affinity toward one another and by restricted receptor expression/distribution in normal tissues.[103] More

Table 3. Examples of Peptides as Ligands for Other Drug Entities*

Peptide Ligands and Analogs	Cytotoxic and Radioactive Agents	References
A. Targeting tumor vasculature		
CNGRC (linear), GNGRG (cyclic)	TNF-α	81
CDCRGDCFC CNGRC	Doxorubicin	82
CRGDCGG	Tachyplesin (antimicrobial peptide)	83
CNGRC	$(KLAKLAK)_2$ (pro-apoptotic peptide)	84
B. Targeting cell-surface hormone receptors		
Somatostain	^{111}In, ^{90}Y, ^{177}Lu	85
Bombesin	^{99m}Tc	86
Gastrin-releasing peptide (GRP)	^{99m}Tc	87
Somatostain, Bombesin, LHRH	Doxorubicin 2-pyrrolino-doxorubicin	88,89
LHRH, LH β-chain (AAs 81–95), HCG fragment	Hecate (membrane-lytic peptide)	90,91

*TNF-α indicates tumor necrosis factor α; LHRH, luteinizing hormone-releasing hormone; HCG, human chorionic gonadotropin.

important, folic acid (MW 441) appears to meet most (if not all) criteria set forth previously for a desirable targeting ligand for proteins and peptides. Regardless of the size, folate-drug conjugates are transported into the cells via receptor-mediated endocytosis. They are internalized and later trafficked into acidic endosomes where some of the folate conjugates will dissociate from the FR as the binding of folate to its receptor is pH-dependent. The receptor internalization efficiency (10% to 25% by 6 hours in cell culture) is not very high since most of the FR does not endocytose, but rather remains stationary on the cancer cell surface.[104] This continuous recycling of only a fraction of cell surface FR may create a challenge for drugs requiring high intracellular concentration to take effect, but it is quite preferable for drugs that require only surface presentation.

To date, examples of folate-targeted protein agents have included (1) protein toxins (momordin, gelonin, *Pseudonomous* exotoxin), (2) penicillin-V amidase (PVA)/doxorubicin prodrug, (3) anti-T-cell receptor (TCR)/CD3 antibodies, and (4) endogenously induced anti-hapten antibodies.[40,105] With suitably controlled chemistry, folate-protein conjugates can be made to preserve the protein's full activity.[12,14] For recombinant momordin and *Pseudonomous* exotoxin, the IC_{50} of their folate derivatives in FR-expressing cells is $<10^{-9}$ M compared with $>10^{-5}$ M for the underivatized toxins.[12,13] Treating cancer cells with

Table 4. Antibody-Targeted Antimitotic Peptides

Targeting Ligands	Cytotoxic Peptides	Tumor Target	References
Anti-CD20	Monomethylauristatin E	B-cell malignancies	98
Anti-CD30	Auristatin E Monomethylauristatin E	Hematological malignancies	99,100
Anti-Lewis Y	Auristatin E Monomethylauristatin E	Carcinomas	99
Anti-E-selectin	Monomethylauristatin E	Prostate cancer	101
Anti-TMEFF2	Auristatin E	Prostate cancer	102

folate-modified PVA followed by a doxorubicin prodrug showed a quantitative and tumor-specific killing of an FR-positive human cancer cell line (KB) but had no effect on an FR-negative cell type (A549).[20] Despite these encouraging results, the tumor uptake of folate-protein conjugates in vivo has been largely disappointing. Low molecular weight folate conjugates are often concentrated in FR-expressing tumors >100 fold over their nontargeted counterparts. In contrast, the enrichment of folate-conjugated bovine serum albumin (BSA) in an FR-positive tumor only marginally exceeds passive targeting (the EPR effect) with 0.46% versus 0.32% of the injected dose of folate-BSA and non-targeted BSA, respectively.[106] This low tumor accumulation may be in part attributable to increased intratumoral pressure and possibility binding site barrier owing to the high affinity of folate conjugates to FR. It is conceivable that after leaving blood vessels, folate-protein conjugates tend to bind avidly to FR on cancer cells directly adjacent to the capillary bed. This creates a real-time obstacle that blocks the diffusion of succeeding protein conjugates to cells deeper within the tumor tissue. For tumor cells growing in suspension, Ward et al. demonstrated a marked uptake of folate-BSA-FITC in tumor cells isolated from the ascitic fluid of ovarian cancer patients.[107]

Folate-targeted immunotherapeutic agents seemed to have better efficacy in live animals. Roy and others[108] have constructed various folate/antibody conjugates specific for the TCR/CD3 complex or the costimulatory CD28 molecule. Intracerebral and/or intraperitoneal administration of folate-scFv and folate-Fab conjugates could promote T-cell infiltration in FR-positive cancer of murine choroid plexus and human KB xenografts. The antitumor response varies depending on the route of administration, tumor location and size, and the activation status of T lymphocytes. Overall, better efficacies were seen with disseminated tumors 0 to 2 days old in the presence of activated lymphocytes generated by adoptive transfer or pretreatment with T-cell activating agents (staphylococcal enterotoxin B, IL-12, CTL-stimulating peptide SIYRYYGL). In collaboration with Dr Philip Low at Purdue University, we have developed an alternative means for targeting endogenous antibodies to FR-expressing tumors, which might shed some light on the issue of targeted delivery systems for protein drugs. In animals with preestablished anti-hapten antibody, we started the targeting process by administrating a small folate-hapten conjugate that was shown to bind FRs on cancer cells and penetrate into tumor mass at high concentrations.[109] As a result of the folate-hapten binding to FR, anti-hapten antibodies were seen to rapidly opsonize the cancer cells, rendering it "marked" for removal by the immune system. Immunofluorescence analysis of tumor sections taken from animals under this condition revealed that anti-hapten antibodies achieved remarkable penetration within tumor tissues.[110] Moreover, using cytokines to assist the tumor-reactive immune cells, established murine tumors (eg, 7-day-old intrapertoneal or subcutaneous tumor) can be completely eradiated by the above protocol, and long-term protective immunity against the same tumor cell line developed in the process.[105,110] While redirecting endogenous antibodies is not a common practice in ligand-targeted protein delivery, it does appear that in order to concentrate a protein in a tumor, it may be easier to first saturate the tumor mass with a low-molecular-weight bispecific ligand that is capable of forming a bridge between the tumor and the protein drug of interest. A nontoxic form of the protein drug is then given systemically, which becomes trapped in the tumor via both the EPR effect and the previously localized bispecific ligand.

CONCLUSION

The enormous potential of protein and peptide drugs has reenergized the scientific community in search of better ways to achieve disease specificity. The effectiveness of any targeted therapeutics largely depends on whether the right drug can be delivered to the right location in sufficient quantities and in a timely fashion. Tumors are notoriously heterogeneous and unstable in terms of their morphology and gene expression. Except for anti-angiogenic proteins and peptides, most ligand-targeted protein drugs or antibody-targeted cytotoxic peptides do not reach active binding sites deep within a solid tumor. Continuing efforts are being made to improve the tumor delivery and retention of macromolecular therapeutics. Strategies to enrich a tumor mass with a macromolecular protein drug may benefit from first enriching the malignant tissue with a small high-affinity bispecific ligand and then permitting diffusive forces to slowly drive the docking of macromolecules recognized by the previously positioned ligand.

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