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PROTEINS AND PEPTIDES AS TARGETING CARRIERS IN ANTICANCER DRUG DELIVERY: A REVIEW

Alemu Tekewe*, Murad Saleh and Meron Kassaye

Department of Pharmaceutics and Social Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia

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Correspondence to Author:

Alemu Tekewe

Department of Pharmaceutics and Social Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia

E-mail: atekewe@yahoo.com

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ABSTRACT

The treatment of cancer is limited by a number of factors including the low therapeutic index of most chemotherapeutic agents, the emergence of drug- and radiation-resistant tumor cells, tumor heterogeneity and the presence of metastatic disease. One of the means to improve the therapeutic index of drugs is by selective or 'targeted' delivery to tumor sites. Tumor-directed therapy has the potential to improve efficacy, by increasing the intratumoral concentration of the targeted agent, and to minimize toxicity by reducing systemic exposure. So far some degree of site-selective delivery has been achieved only with "targeting homing drugs" that specifically recognize their pharmacological target. The specificity of delivery using nanoparticles was initially a coincidental property, active targeting has now become a central concept in cancer therapeutic research. This concept has been developed into practical application using a variety of tumor targeting ligands. This review briefly summarizes the ever increasing evidence to the use of proteins such as monoclonal antibodies (MAbs), bispecific antibodies (BsAbs), Affibody molecules, albumin, transferrin and peptides such as stable microbial toxins and cell penetrating peptides (CPPs) as innovative tumor targeting ligands in anticancer drug delivery systems.

INTRODUCTION: Cancer is one of the most wide-spread diseases of modern times; with an estimated increase in the number of patients diagnosed worldwide, from 11.3 million in 2007 to 15.5 million in 2030 [1].

Despite the significant progress in the development of anticancer technology, there is still no common cure for patients with malignant diseases. In addition, the long-standing problem of chemotherapy is the lack of tumor-specific treatments. Traditional chemotherapy relies on the premise that rapidly proliferating cancer cells are more likely to be killed by a cytotoxic agent. In reality, however, cytotoxic agents have very little or no specificity, which leads to systemic toxicity, causing undesirable severe side effects such as hair loss, damages to liver, kidney, and bone marrow [2, 3]. It is therefore of importance to develop novel technologies that can be used for targeted drug delivery to tumors and thereby improve the therapeutic index of the drugs by increasing therapeutic drug concentrations to the target tumor tissue and/or by reducing systemic drug distribution to minimize the severe and harmful toxic effects on normal organs [4].

In the last three decades, various drug delivery protocols and systems such as liposomes [4], micelles, dendrimers, various polymeric based systems [5], nanostructure [6] and nanoparticles [7], MAbs and other protein based carriers, peptides [8], polyunsaturated fatty acids, folic acid, hyaluronic acid and oligopeptides [2] have been explored.

Among these carrier systems, proteins and peptides as targeting carriers in anticancer drug delivery holds great promise because of their selective binding affinity, tissue penetration capacity and internalizing capacity by cancer cells. Further more, some protein and peptide-based carriers are nearly invisible to the immune system and are expected to cause minimal or no side effects [8].

In addition, recent advances in protein engineering, protein nanoparticle technology and the advances in combinatorial peptide library technology have made it possible to generate high-affinity proteins and peptides that may be used as ligands for the development of targeted anticancer strategies [8, 9]. For example, MAbs [8], CPPs [10], Affibody molecules [11], albumin [12], BsAbs [13], transferrin [14] and stable toxin peptides [15] are some of the new classes of transmembrane delivery vectors with high pharmaceutical potential.

Generally, the development of novel drugs and delivery systems with novel mode of actions and high cancer cell selectivity are crucial in order to battle anticancer drug resistance and reduce normal cell cytotoxicity [16]. Now a days significant research efforts have been directed towards targeting cancer drugs to tumors using specialized drug carriers, and protein- and peptide- based carriers have become an important component of these targeting approaches [3]. It is likely that the use of such drug carriers can improve the pharmacological properties of conventional chemo-therapeutics by altering drug pharmacokinetics and biodistribution [5, 16].

This review briefly describes some of the recent advances in using proteins and peptides as targeting carriers in anticancer drug delivery, with particular emphasis on those proteins and peptides that are currently in use or under investigations and may soon become part of the novel drug targeting approaches in the therapeutic arsenal to combat cancer in a more efficient way.

Proteins as Anticancer Drug Targeting Carriers: The idea of developing a drug that selectively destroy disease cells without damaging healthy cells was proposed by Paul Ehrlich, almost a century ago; he called his hypothetical drug the “magic bullet” [17-19]. Thereafter, over the past several decades, many scientists have focused their attention on the development of ideal drugs that specifically target the site of action. Although little progress has been made in this field, the advent of nanomedicine and our understanding of cellular and molecular biology have opened new avenues to transform the Ehrlich's concept into clinical reality [18].

A concept of site-specific drug-delivery systems was formed and, according to this concept, a drug would be attached to a carrier that would take the ‘pay-load’ (the drug) to the target (attached to the carrier via a targeting ligand) and release it at the target site. The practical realization of this concept has fascinated and eluded scientists ever since [19].

The choice of the carrier molecule for targeted drug delivery system is of high importance because it significantly affects the pharmacokinetics and pharmacodynamics of the drugs [18].

Over the past four decades intensive efforts have been made to design novel carrier systems that are able to deliver the drug more efficiently to the target tumor site. A wide range of materials, such as natural or synthetic polymers [18, 20], lipids, surfactants,[18] dendrimers [18, 20], polysaccharides [18] cells, micro-spheres, nanospheres, liposomes [20-22], polymer micelles [20, 22-24], nanoparticles [20-22, 25], inorganic nanoparticles, carbon nanotubes [21], lipoproteins [24], antibodies and proteins [22] have been extensively investigated for passive and/or active targeting of the tumor tissues. Among these, protein-based carrier systems have received increasing attention because of their outstanding physical and biological properties for tumor targeting [17].

Proteins are a class of natural molecules that have unique functionalities and potential applications in both biological as well as material fields. Nanomaterials derived from proteins, especially protein nanoparticles are biodegradable, non-antigenic, metabolizable and can also be easily amenable for surface modification and covalent attachment of drugs and ligands. Because of the defined primary structure of proteins, the protein-based nanoparticles may suggest various possibilities for surface alteration and covalent drug attachment.

In the last one decade, active research was focused on the preparation of nanoparticles using proteins for targeted delivery of anticancer drugs [26]. The protein nanoparticles can deliver the therapeutic drugs to the tumor by passive or active targeting. Passive targeting of nanoparticles to tumors occurs by the modulated vasculature, which allows nanocarriers to extravasate through gaps in the endothelium.

The entry of the particles to the interstitial space, associated with poor lymphatic drainage from the tumor, results in higher retention times of nanoparticles in the tumor than in normal tissues, in a process known as the enhanced permeability and retention (EPR) effect [27-29]. Significant increases in drug accumulation in the tumor tissue by the EPR effect can reach 10- fold or higher concentration with drug-loaded nanoparticles compared to free drug. Active targeting requires the use of targeting moieties, such as antibodies or receptor ligands or other proteins and peptides, conjugated to the surface of the nanocarrier systems for their delivery enhancement [27]. Some of the proteins that have promising applications for passive and/or active targeting of tumors in treating cancers using chemotherapeutic agents are briefly discussed as fellows;

1. **Monoclonal antibodies for Targeted Anticancer Drug Delivery:** Advances in understanding of the molecular mechanisms underlying the development and progression of cancer have resulted in the discovery of new therapeutic interventions that target specific molecular abnormalities [30].

   About 55 years ago, it was suggested that antibodies could specifically target malignant cells *in vivo*, but it was the discovery of hybridoma technology by Kohler and Milstein in 1975 that paved the way for the development of antibody based cancer therapy [30, 31]. The hybridoma technology has enabled the development and production of MAbs in large scale that can be specifically directed against each particular cellular antigen [32, 33]. In this procedure mice are mostly immunized with tumor cells or with a purified tumor antigen.

After fusion of spleen cells from the immunized mouse with myeloma cells, a hybridoma cell clone can be selected to produce a MAb with the desired antigen specificity [32]. The advents of molecular biology techniques and allied technologies during the past two decades have greatly facilitated the genetic manipulation, recombinant production, identification and conjugation of antibody fragments. These have improved the capacity to design and generate MAbs that specifically target and subsequently eliminate cancer cells.

The implementation and refinement of genetic fusion and recombinant expression techniques have led to the development of a large variety of engineered MAb molecules for research, diagnosis and therapy [30]. In addition *in vitro* routes to high affinity MAbs can be explored by using phage display libraries [32]. In general, the advances of such molecular techniques and their applications for the generation of antibodies to tumor associated antigens gave rise to early hopes that such antibodies would be the sought-after 'ideal anti-cancer drug' [31]. More than two decades of development has led to the therapeutic promise of MAbs for cancer and other diseases such as cardiovascular diseases, infectious diseases and immune disorders being realized [33, 34].

Nowadays, more than 20 MAbs have been approved for use in many indications, including cancer [35]. The promise offered by MAb-based therapies for treatment of cancer has begun to be realized with the approval of an anti-CD20 MAb, rituximab (Rituxan, IDEC Pharmaceutical Corp, San Diego, CA), for treatment of non-Hodgkin's' lymphoma and B-cell chronic lymphocytic leukaemia, an anti-HER2 MAb, trastuzumab (Herceptin, Genentech, CA) for treatment of metastatic breast carcinoma [33, 36-38] and cetuximab for colorectal cancer [31].

So far, 6 unconjugated antibodies and 3 immuno-conjugates approved for use in the United States in a variety of cancers, with a considerable number of new agents in advanced clinical trials [39, 40]. Novel MAbs that target various receptors are also in preclinical trials [39, 41].

How MAbs actually induce anti-tumor effects is not fully understood. Initially the major mechanisms were thought to be T-cell mediated antibody-dependent cellular cytotoxicity (ADCC) and/or opsonisation and complement dependent lysis as shown in figure 1 [33, 36, 39, 42, 43]. However, MAbs that target proteins involved in cell signaling, as shown in **figure 1**, have proven more effective and have multiple actions [33, 36, 39, 41]. The mechanisms of action of trastuzumab, for example, include receptor down regulation, prevention of heterodimerization, initiation of cell cycle arrest, prevention of receptor cleavage by proteases and inhibition of angiogenesis. In addition, trastuzumab appears to restore sensitivity to taxanes and other DNA damaging agents, probably by inhibiting anti-apoptotic signals [33, 41].

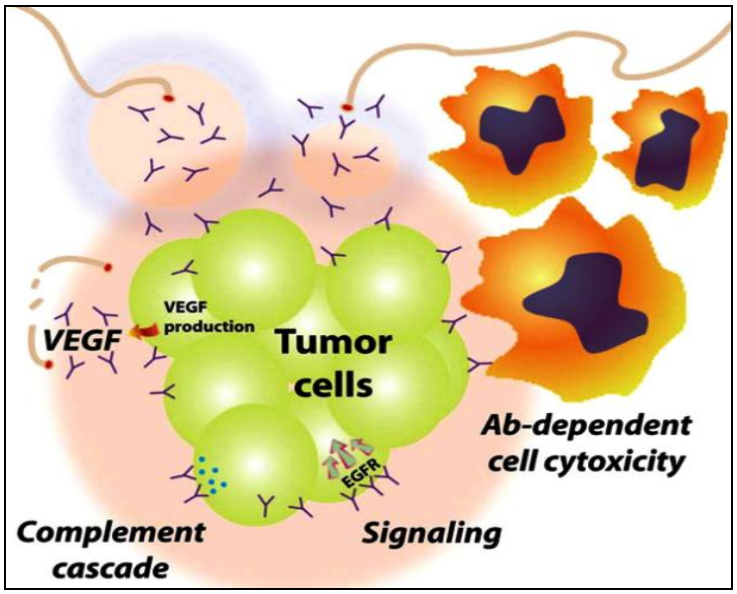

FIGURE 1: MECHANISMS OF ACTION FOR UNCONJUGATED ANTIBODIES [39]

Even though "naked" MAbs can exert their therapeutic efficacy through multiple mechanisms, often their activity is not sufficient to produce a lasting benefit. Hence, several strategies have been employed to enhance their activity [35, 37].

Antibody–drug conjugates represent one approach, where the ability to harness MAb specificity and target the delivery of cytotoxic agents such as radionuclides, chemotherapeutic drugs or toxins to the tumor cells in order to elicit a tumor specific cytotoxic effect [33, 34, 42].

This strategy increases effectiveness and reduces non specific toxicities of radio-isotopes, toxins, drugs or enzymes because the antibodies can selectively bind with antigen bearing cells and deliver their "payloads" directly to tumor cells [35, 44]. For example, radiolabeled MAbs directed against tumor associated antigens or tumor surface antigens selectively concentrate the radiolabel at the site of the tumor, allowing imaging of the primary tumor and/or metastases or site selective delivery of radio-isotopes for therapeutic use [44].

Several MAb–drug conjugates have displayed pronounced activities in preclinical cancer models, and there are now three approved antibody–cytotoxin conjugates for cancer therapy: MylotargTM (gemtuzumab-ozogamicin), Zevalin TM (ibritumomab-tiuxetan), and BexxarTM (tositumomab). Research surrounding the critical parameters for therapeutic success has suggested that highly potent drugs are required for MAb-based delivery strategies, and the linker used to attach the drug to the MAb should be highly stable in circulation [45].

Conjugation also has an effect on the biodistribution of the drug, sparing normal tissue exposure to the cytotoxic agent and allowing the use of potent agents that would prove too toxic for systemic use. Optimization of a variety of antibody-drug conjugation parameters has recently met with considerable success for the targeted delivery of cytotoxic payloads to tumor cells [35, 45].

Different strategies could be defined for targeted MAb-drug conjugates. In the first strategy, drug could be bound to the antibody in such a way that drug was released from the conjugate by a non-specific process, e.g., hydrolysis or dissociation, leading to a half-time for drug release.

In the second strategy, drug could be targeted to the tumor tissue, and released by mechanisms in the extracellular environment. The tumor environment is known to be more acidic than normal tissue and also contains amounts of cathepsins and other proteases.

This strategy has the advantage that it does allow some degree of bystander effect, i.e., killing of non-antigen-bearing tumor cells, to take place, but may not deliver drug as rapidly or as specifically to the tumor cells [46].

In the third strategy, macromolecules would be endocytosed into cells, and drug released from the lysosomal degradation of the macromolecule. This is the major mechanism for which most chemoimmuno-conjugates have been designed. This has the advantage that only cells bearing the relevant antigen can be killed following endocytosis of the conjugate but conversely suffers from the problem that cells not bearing the relevant antigen, or which do not endocytose the antigen are not killed. However, it is likely that the drug release mechanisms employed for this route may also allow some release of drug from the extracellular compartment through the proteases and slightly lowered pH [31, 37, 38, 46, 47].

The fourth strategy is the so called antibody directed enzyme prodrug therapy. In this approach, an enzyme is coupled to a target cell directed antibody. After administration, the enzyme-MAb complex is allowed to bind to its target. Subsequently, a non-toxic, prodrug is administered. Upon exposure to the enzyme-MAb complex, the active drug is enzymatically cleaved from the prodrug and released locally at high concentrations. This strategy is one of the various tumor targeting approaches to improve the efficacy of anticancer chemotherapy by reducing the adverse side effects and damage to normal tissues associated with systemic dug delivery and therapy [48, 49-51].

This therapeutic approach seems to be justified in an adjuvant setting for the treatment of minimal residual disease or leukaemia or after surgery of the primary tumor to kill possible circulating tumor cells. However, by using this approach few clinical responses were observed in the treatment of solid tumors due to heterogeneous and low uptake of conjugates of anticancer agents and antibody or pro-antibody fragments. To overcome the problem of delivery of MAbs for the therapy of larger tumor masses, smaller fragments such as single chain variable domain fragments (ScFv) and more easy accessible target cells such as tumor vascular endothelial cells have been studied [48, 52, 53]. More over, it is clear that application of the advanced and new molecular technologies to refine the macromolecular structure of the MAbs to maximize tumor targeting and penetration will be of great utility in improving the efficacy of antibody based cancer immunotherapy [54, 55].

2. **Bispecific antibodies for Targeted Anticancer Drug Delivery:** To overcome dose limiting toxicities and to increase the efficacy of cancer therapy, so far a number of strategies have been tried for selectively targeting drug molecules towards tumor cells. Many of these strategies exploit the specificity of tumor associated antigen recognition by MAbs or MAB-drug conjugates[48]. However, low efficacy of “naked” MAbs as cancer monotherapeutic agents and problems associated with chemical conjugation of cytotoxic agents with MAbs, such as reduction of antigen binding activity, inconsistency of drug loading, aggregate formation, and low protein yields, have led several groups to construct ligands that can combine an antigen binding site with one that recognizes a cytotoxic agent [56]. The recent breakthroughs in recombinant DNA technology, the increased number of identified disease targets as the result of the completion of human genomic map project, and a better understanding of the mechanism of human immune system has helped scientists to develop BsAbs [52].

   BsAbs are constructs that have two specificities, one directed at the effector cells and the other directed at the target cell. Their development arose out of the need for targeting immune cells to tumor antigens[13]. The development of BsAbs elicited possibilities to combine tumor cell and immune effector cell specificities in a single antibody molecule [48]. Through BsAb mediated cross linking of tumor cells and immune effector cells such as cytotoxic T lymphocytes, natural killer cells, neutrophils and monocytes/macrophages, the effector cells were able to redirect their cytolytic activity towards the tumor cells in a highly efficient manner [48, 52].

Cross linking and cytolysis are induced irrespective of the effector cell's intrinsic specificity and major histocompatibility complex (MHC) expression by the tumor cell [48]. For example, BsAbs direct cytotoxic T cells to mediate tumor cell lysis regardless of their initial antigen specificity. In addition, the interaction between redirected cytotoxic T cells and tumor cells is independent of MHC antigens, so that the cytotoxicity is not affected by MHC alteration or down regulation on the tumor targets, by which cancers effectively evade the immune attack [48, 57].

BsAbs can be conventionally generated by hybrid-hybridoma method, or by chemical conjugation [13, 58]. Through chemical conjugation means, BsAbs were generated by chemical cross linking of respective F(ab`) fragments of target and trigger molecule antibodies [58]. In the early 1980s, the production of BsAbs was also successfully via fusion of two MAb producing cell lines (or hybridomas). Using this fusion technique, one is able to obtain a hybridoma cell line secreting the BsAb of choice.

Due to variation in genetic recombination, however, the fusion product will consist of cell lines synthesizing a variety of proteins in addition to the desired BsAb producing cell lines in the correct format. Therefore one needs appropriate selection methods to isolate the particular BsAb producing cell line [48]. Both of these techniques have their disadvantages for clinical uses due to high cost, large molecular size, instability and immunogenicity [57, 58].

In the 1990s, advances in molecular genetics and protein engineering and the expansion of knowledge on recombinant DNA technology led to the development of a new generation of recombinant BsAbs that are suitable for *in vivo* application [48, 58]. Promising new formats for recombinant BsAbs include single-chain bispecifics, bispecific diabodies and bispecific minibodies containing individual constant domains of conventional antibodies. All these constructs can be made from humanized or even fully human antibodies [57, 58].

One of the more notable recent achievements has been the design of tandem BsAbs, which behave as tetrabodies and thereby comprise two bivalent components that provide both high targeting avidity and receptor activation [57]. In general, while BsAb preparation by chemical synthesis or hybridoma fusion yield relatively high amounts of product, BsAbs produced by recombinant DNA technology are better defined. This latter technology will require up scaling of BsAb protein production for future *in vivo* experimental and clinical evaluation [48].

BsAbs have drawn considerable attention from the research community due to their unique structure against two different antigens. The two-arm structure of BsAbs allows researchers to place a therapeutic agent on one arm while allowing the other to specifically target the disease site [52, 59]. The therapeutic agent can be cytotoxic drugs, toxins, enzymes, DNA, prodrugs, antivascular agents, cytokines, viral vectors, or radionuclides [13, 48, 52].

Furthermore, BsAbs may redirect the cytotoxicity of immune effector cells towards the diseased cells or induce a systemic immune response against the target. BsAbs have been also introduced in cancer vaccine development. Some of these exciting explorations have already been expanded to redirecting cytotoxicity to tumor cells, HIV and other infectious diseases; targeting enzymes to achieve site-specific activating anti-cancer prodrugs and delivering antigen specifically to antigen-presenting cells as vaccines [52].

BsAbs have been also exploited in a large variety of applied technologies such as immuno-histochemistry, enzyme immunoassays and for studying cell–cell interactions [48]. Due to its great potential as new anticancer drug targeting ligand and for new therapeutic applications, enormous research efforts should be devoted to this area.

3. **Affibody molecules for Targeted Anticancer Drug Delivery:** Affibody molecules (Affibody) are small and robust affinity ligands based on the three-helical-bundle Z domain, which is a stabilized variant of the B domain of staphylococcal protein

A [11, 60]. They are not related to and do not share sequence or structural homology with antibodies [60]. The Z domain was chosen as the starting point for the construction of novel binding proteins because this small protein (58 amino acids, approximately 6.5 kDa) was known to have excellent biophysical properties—including high melting temperature, reversible and rapid folding, a binding surface as large as that of an antibody, high solubility in aqueous solutions. The exceptional nature of the Z domain scaffold is further highlighted by the shortest folding time yet reported for a protein, that is, 3 μs [60-62].

Unlike MAbs that may be generated by immunization of laboratory animals combined with hybridoma technology, isolation of new affibody molecules based on non-immunoglobulin scaffolds is performed using synthetic combinatorial libraries and *in vitro* selection systems (e.g. phage display technology) [61]. Affibody-based scaffolds fold spontaneously in physiological conditions and they can be also produced by recombinant DNA technology using bacterial cells such as *Escherichia coli* (*E. coli*) as expression hosts [63, 64].

Affibody molecules are typically selected from phage-displayed combinatorial libraries, where 13 surface-exposed amino acid residue positions on helices 1 and 2 have been randomized to create large molecular repertoires [61, 63, 64]. They can be fused in tandem, should bivalent or bispecific constructs be desired. As affibody molecules are devoid of cysteines, they allow homogenous site-specific labeling using maleimide chemistry after the introduction of a single cysteine for such labeling purposes [63].

The robust scaffold enables labeling of Affibody molecules in a variety of conditions including reducing environment, broad range of pH and elevated temperatures without loosing binding properties. Site-specific labeling of Affibody molecules made by peptide synthesis can be also achieved by coupling a chelator to N-terminus in the last synthesis step [64]. Affibody molecules are a class of engineered affinity proteins with proven potential for therapeutic, diagnostic and biotechnological applications. Their ability to selectively and with high affinity bind a given molecular structure is an essential key feature for *in vitro* and *in vivo* diagnostics, for basic research and for many biotechnological applications [61, 64].

They have been successfully tested for targeted diagnostic utility in cancer patients with human epidermal growth factor receptor 2 (HER2) - expressing metastases and they are used as affinity ligand in an IgG affinity purification column. While many different binding members have been explored for biotechnological use, several Affibody molecules with different specificities have been used for *in vivo* purposes [61].

Affibody molecules with nanomolar and picomolar affinities have been produced by recombinant DNA technology or selected from phage-displayed combinatorial libraries to a large range of targets, including insulin, fibrinogen, transferrin, IL-8, gp120, CD28, human serum albumin, IgA, IgE, IgM, HER2, epidermal growth factor receptor (EGFR), tumor necrosis factor α (TNFα) and amyloid-β (Aβ) peptide [11, 60, 61].

Multimeric Affibody molecules (i.e., head-to-tail gene fusions of two or more Affibody molecules), bispecific Affibody molecules i.e., fusion of two Affibody molecules having separate target specificities and fusions of Affibody molecules with other proteins and toxins have been shown to be functionally active. Since the Affibody scaffold lacks cysteines, homogenous site-specific modifications are possible by the introduction of a unique cysteine. This has been done to achieve site specific labeling with, for example, different radionuclides and fluorescent dyes.

Thus, Affibody molecules have been shown to be amenable for a wide range of additional modifications, including fusions at the N- or the C-terminus [60]. More recently, different groups have investigated Affibody molecules as alternatives to antibodies for nanoparticulate anticancer drug delivery. This scaffold has excellent features like their stability, solubility, their intrinsic small size, fast folding and simple but robust non-cysteine containing structure as an affinity ligand and can

be designed to bind with high affinity to any given target protein [61, 62]. As only 13 amino acid positions differ between binding members specific for different receptors and proteins, much of the knowledge and techniques on modulation one Affibody molecule can be applied to another [61].

Thus, such intrinsic features enable one to design and characterize novel procedures for developing different Affibody molecules for targeted delivery of therapeutic payloads to cancer cells. Most early work on *in vivo* targeting has been done with HER2 and later EGFR-targeting Affibody molecules. For example, HER2-binding Affibody has several merits as a targeting ligand owing to a) its small size (5.8 kDa), b) ease of conjugation of functional domains away from the active site c) ability to promote receptor-mediated endocytosis, and d) high stability in vitro and in vivo and importantly, non-toxic to cells [61, 65].

4. **Albumin based Drug Targeting in to Tumor Cells:** Albumin is the most abundant plasma protein (35–50 g/L human serum) with a molecular weight of 66.5 kDa. Like most of the plasma proteins, albumin is synthesized in the liver where it is produced at a rate of approximately 0.7 mg/h for every gram of liver (i.e. 10-15 g daily). It is an acidic, very soluble protein that is extremely robust: it is stable in the pH range of 4-9, soluble in 40 % ethanol, and can be heated at 60 °C for up to 10 h without deleterious effects [12, 66].

Commercially, albumins are obtained with significant quantities from egg white (ovalbumin), bovine serum (bovine serum albumin), and human serum (human serum albumin, HSA) and also available from soybeans, milk and grains [67]. The HSA that exhibits an average half-life of 19 days can be used for treating shock, burns, hypoalbuminemia, surgery or trauma, cardiopulmonary bypass, acute respiratory distress and hemodialysis. As an alternative to blood derived albumin, recombinant HSA (Recombumin) has been developed and is a genetically engineered protein expressed in yeast cells that has shown comparable safety, tolerability, pharmacokinetics and pharmacaodynamics to native HAS [12, 66, 67].

In general, the multifold functions and binding properties of HSA as well as its preferential uptake in tumor and inflamed tissue, its ready availability, its biodegradability, biocompatibility, its effective drug loading capacity, water solubility, and its lack of toxicity and immunogenicity make it an ideal candidate for drug delivery [12, 68, 69]. More over, HSA provides several functional groups on the surface which can be easily used for surface modification [69].

Albumin is emerging as a versatile protein carrier for drugs with high binding affinity for albumin to either improve the pharmacokinetic profile and bioavailability of drugs such as peptides and antibody moieties or to exploit the targeting property of albumin for inflamed or malignant tissue [12, 66]. This macromolecular carrier has been shown to be biodegradable, nontoxic, metabolized *in vivo* to produce innocuous degradation products, non-immunogenic, easy to purify and soluble in water allowing ease of delivery by injection and thus an ideal candidate for nanoparticle preparation.

Albumin-based nanoparticle carrier systems represent an attractive strategy, since a significant amount of drug can be incorporated into the particle matrix because of the different drug binding sites present in the albumin molecule. Due to the defined albumin primary structure and high content of charged amino acids (e.g. lysine), albumin-based nanoparticles could allow the electrostatic adsorption of positively or negatively charged drug molecules without the requirement of other compounds such as surfactants or polymeric materials.

In addition, albumin nanoparticles can be easily prepared under soft conditions by coacervation, controlled desolvation or emulsion formation. They show a smaller size (50 to 300 nm) compared to microparticles and, in general, better controlled release properties than liposomes which may improve patient acceptance and compliance [67, 70]. Furthermore, albumin is thought to facilitate the endothelial transcytosis of unbound and albumin-bound plasma constituents to the extravascular space. This process is initiated by the binding of

albumin to the endothelial cell surface to the 60-kDa glycoprotein (gp60) receptor (albondin), which in turn results in the binding intracellular protein (caveolin-1) by gp60 and invagination of the cell membrane to form transcytotic vesicles, referred to as caveolae.

This efficacy conferred by the use of an albumin carrier is supported by the findings of several clinical studies, for example, on AlbunexTM and AbraxaneTM [70].

Over the past decades, albumin has emerged as a versatile carrier for targeting therapeutic and diagnostic agents, primarily for diagnosing and treating cancer, diabetes, rheumatoid arthritis and infectious diseases [71]. Market approved products include the taxol albumin nanoparticle Abraxane® for treating metastatic breast cancer which is also under clinical investigation in further tumor indications [66, 68, 71, 72], fatty acid derivatives of human insulin or the glucagon-like-1 peptide (Levemir® and Victoza®) for treating diabetes [66, 71], and 99mTc-aggregated albumin (Nanocoll® and Albures®) for diagnosing cancer and rheumatoid arthritis as well as for lymphoscintigraphy [71].

Other recent applications of HSA have demonstrated some advantages as a natural and therefore biocompatible and biodegradable carrier to construct targeted cytotoxic conjugates with apoptosis-inducing drugs. In these cases the albumin-based targeted drug delivery system has increased the disease tissue/normal tissue drug concentration ratio [72]. In addition, some albumin-based or albumin-binding drugs are in clinical trials for treatment of cancers, diabetes, rheumatoid arthritis and vascular diseases as shown in table 1 below [71].

TABLE 1: OVERVIEW OF ALBUMIN-BASED DRUGS THAT HAVE REACHED MARKET APPROVAL OR ARE IN DIFFERENT STAGES OF CLINICAL DEVELOPMENT [71]

Indication	Phase 1	Phase II	Phase III	Market Approval
Diabetes				Levemir®
Diabetes				Victoza®
Diabetes	CJC-1134-PC			
Oncology				Abraxane®
Oncology		INNO-206		
Oncology	MM-111			
Oncology		AFL-HSA		
Oncology	ZHER2:342			
Rheumatology		ATN-103		
Oncology Rheumatology				Nanocoll®
Oncology Rheumatology				Albures®
Vascular disease				Vasovist®

Considering the commercial success of products that use albumin as a drug carrier and the ongoing clinical trials as well as due to the many diverse technologies of improving the pharmacokinetic profile and drug targeting of therapeutic and diagnostic peptides, antibody fragments, as well as low-molecular weight drugs that include peptides, synthetic and natural products and even simple molecules such as nitric oxide, albumin is attracting the interest of biotech companies as well as of large pharmaceutical companies, and it is likely that the ongoing pipeline development will move further albumin-based drugs into the clinical setting [71].

5. **Transferrin as Drug Targeting Carriers in to Tumor Cells:** Transferrins are a family of homologous iron-binding glycoproteins that are found in mammals, marsupials and fish, as well as in insects and other invertebrates [73, 74]. They are monomeric proteins of 76-81 kDa, depending on the extent of glycosylation, and consist of two structurally similar lobes (termed the N- and C-lobes) connected by a short peptide linker. Each lobe contains a single iron-binding site [73]. Human transferrin is a glycoprotein that contains 679 amino acid residues and has a molecular weight of ~79 kDa [14, 75].

The molecule is stabilized by 19 intra-chain disulfide bonds and is protected by three carbohydrate side chains of which two are N-linked (Asn-413 and Asn- 611) and the third is O-linked (Ser-32) [80]. It is synthesized predominantly by hepatocytes [75, 76]. Other tissues expressing transferrin include sertoli, ependymal, oligo-dendroglial , metastatic melanoma cell lines and human breast cancer cell lines [75].

Transferrin has been detected in various body fluids including plasma, bile, amniotic, cerebrospinal, lymph and breast milk [75, 76]. Plasma concentration of transferrin is stable from birth, ranging from 2 g l^{-1} to 3 g l^{-1}, and the *in vivo* half-life of this protein is about eight days. The level of transferrin is important for healthy growth with levels below 0.1 g l^{-1} associated with an increased incidence of infection, growth retardation and anemia [75].

Transferrin is the principal iron (Fe3+) - transporting protein of the body, binding circulating iron and transporting it to a range of cell types [14, 73, 76]. Iron is essential for a number of cellular functions including DNA synthesis, electron transport, [77] metabolism and respiration [78]. Iron is also a required co-factor for many enzymes that catalyze a wide variety of key metabolic processes, including hemoglobin synthesis in erythroid cells and oxygen transport [78, 79].

In humans and other higher animals, transport of iron has been observed to be predominantly receptor mediated, usually in the form of iron-bound transferrin and this process is triggered by the binding of iron-bound transferrin to specific transferrin receptors (TfRs) on the cell surface [29, 76, 80]. Transferrin binds to at least two distinct types of TfRs, designated TfR1 and TfR2 [80]. The TfR1 (also known as CD71), a type II transmembrane glycoprotein found as a homodimer (180 kDa) on the surface of cells, is a vital protein involved in iron homeostasis and the regulation of cell growth [28, 78]. The TfR1 monomer contains a large extracellular C-terminal domain, a single-pass transmembrane domain, and a short intracellular N-terminal domain [78].

The TfR1 is ubiquitously expressed at low levels on a range of normal cells, except for mature erythrocytes and terminally differentiated cells [77, 78, 79] and expression is increased on cells with a high proliferation rate or on cells that require large amounts of iron. Little or no TfR1 expression has been detected on pluripotent hematopoietic stem cells, while late erythroid and myeloid progenitor cells demonstrate TfR1 expression [78].

A second TfR (TfR2) was identified and has a 25-fold lower affinity for transferrin than TfR1. The human TfR2 is expressed as two transcripts (α-TfR2 and β-TfR2), with α-TfR2 expressed predominantly on liver cells and enterocytes of the small intestine, which is not regulated by intracellular iron levels, and β-TfR2 expressed at low levels on a variety of cell types [75, 78, 79].

Moreover, TfR2 transcript is found to be highly expressed in erythroid precursor cells, where as the protein is not expressed at any stage of normal erythroid differentiation. As TfR2 is able to bind transferrin and internalize iron, even if with a lower affinity compared to TfR1, it was initially considered as a second mediator for iron uptake [79].

Generally, transferrin, which is a monomeric glycoprotein (apo-transferrin), can transport one (monoferric transferrin) or two (diferric transferrin) iron atoms with the help of both TfR1 and TfR2. Diferric transferrin has the highest affinity for the TfR and is 10- to 100-fold greater than that of apo-transferrin at physiological pH [78, 81]. Diferric-transferrin binds to the TfR on the cell surface and the transferrin–TfR complexes are internalized in clathrin-coated pits through receptor-mediated endocytosis.

Upon maturation and loss of the clathrin coat, the endosome is acidified, and iron is released from transferrin and then transported to the cytosol by the divalent metal transporter [78, 80, 82] and plays a key role in cellular growth and proliferation and are also used as a cofactor by heme and ribonucleotide reductase or stored in ferritin [80, 81].

The apo-transferrin–TfR complex is then recycled through exocytic vesicles back to the cell surface where apo-transferrin is released into extracellular space to recruit further Fe3+ ions. The TfR is constitutively recycled back to the cell surface independently of transferrin binding [73, 78, 80]. The mechanism of iron transport and uptake via the transferrin-TfR transport system has the potential to be exploited for site-specific delivery of various therapeutic metal ions, drugs, proteins and genes.

Of particular interest are cells that over express TfR. Transferrin is normally only 30% saturated with iron in the body. At least 30 other metal ions can also bind to transferrin. Therefore, it is possible to use transferrin to transport other metals around the body, in particular, gallium (Ga^{3+}) and indium (In^{3+}) can be transported by transferrin. The cellular uptake of Ga^{3+} occurs mainly via the transferrin–TfR mechanism and thus it concentrates in tissues expressing high levels of TfR, such as tumors. It is for this reason that [67] Ga^{3+}, a low-energy gamma-emitting radionuclide, has widespread use as a diagnostic technique for many malignancies [66, 75].

Despite its ubiquitous expression, TfR is expressed on malignant cells at levels several fold higher than those on normal cells and its expression can be correlated with tumor stage or cancer progression [14, 28, 77, 78, 82]. In addition, studies have also suggested that TfR may play a role in cellular signaling and proliferation stimuli [77]. The high levels of expression of TfR in cancer cells, which may be up to 100-fold higher than the average expression of normal cells, its extracellular accessibility, its ability to internalize, and its central role in the cellular pathology of human cancer, make this receptor an attractive target that can be exploited as a “Trojan Horse” for the delivery of cytotoxic agents for cancer therapy [28, 77, 78, 81, 83, 84].

In fact, the TfR can be successfully used to deliver cytotoxic agents into malignant cells including chemotherapeutic drugs, cytotoxic proteins, peptides, genes or high molecular weight compounds including liposomes, viruses, or nanoparticles as shown in **figure 2** [78, 85].

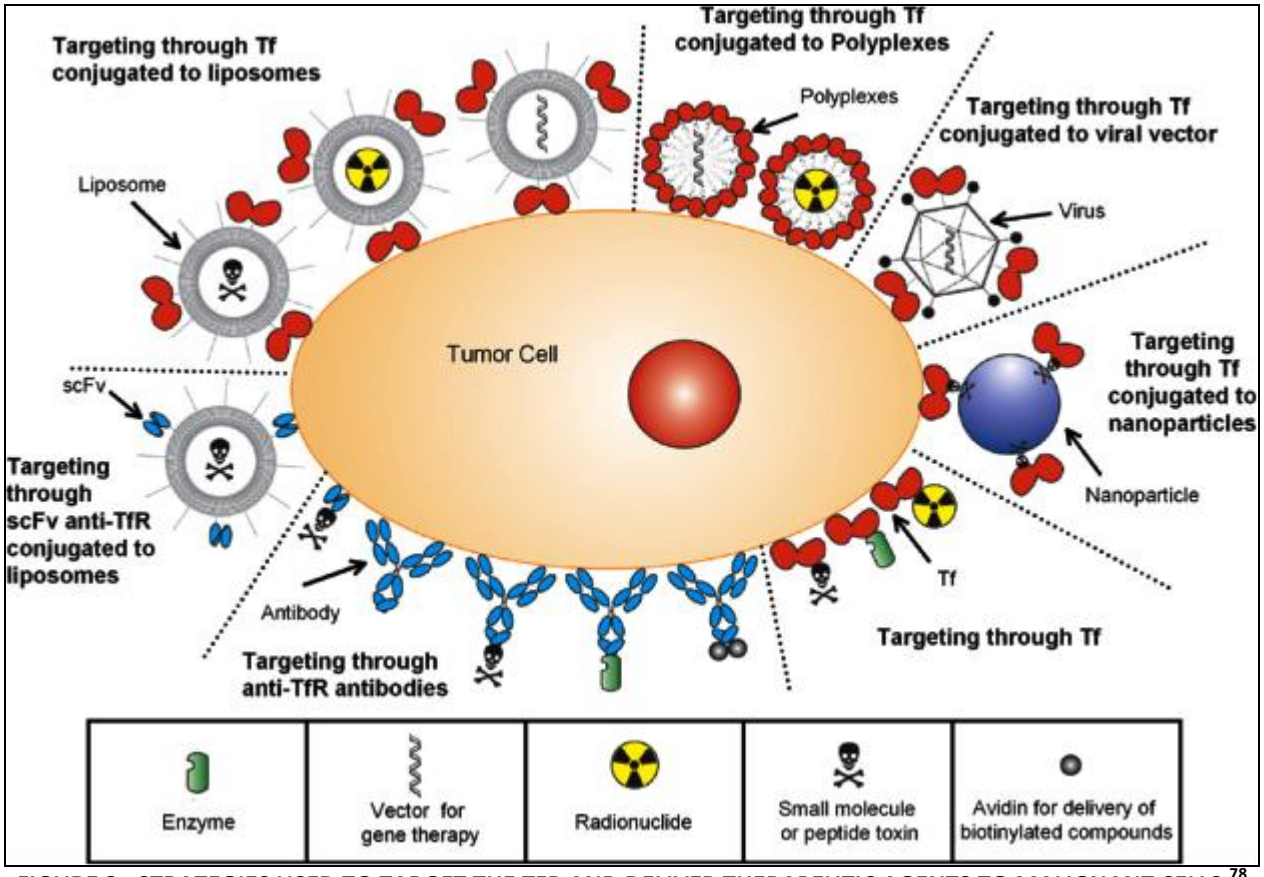

FIGURE 2: STRATEGIES USED TO TARGET THE TFR AND DELIVER THERAPEUTIC AGENTS TO MALIGNANT CELLS [78]

It is now widely accepted that targeted drug delivery using the transferrin-TfR pathway holds promise as ideal targets for cancer therapy, especially for malignancies that are refractory to conventional therapy such as brain tumors [14, 73].

Transferrin can be conjugated with chemotherapeutic drugs, cytotoxic proteins and peptides such as bacterial toxins and plant toxins, DNA, oligonucleotides, short inhibitory RNA (siRNA) and enzymes [27, 86].

Some of the expected advantages of such transferrin conjugates are a preferable tissue distribution, prolonged half-life of the drug in the plasma and controlled drug release from the conjugates [80, 87]. However, cytotoxicity of transferrin conjugates can be blocked by native transferrin. Because high levels of circulating free transferrin in the blood may interfere with the effects of the transferrin conjugates leading to decreased therapeutic efficacy. More over, the transferrin conjugates have the potential to interact with both TfR1 and TfR2 (which is highly expressed in the liver) and they may be particularly toxic in certain cases to liver cells in addition to the targeted malignant cells [27].

Such circumstances and several other factors associated with delivery of payloads through the transferrin-TfR path way make the clinical applications of transferrin conjugates very limited. It is highly possible, however, that rapid developments in material science, pharmaceutical science, protein engineering and biology will allow the fundamental research in this area to be translated into clinical applications, particularly in the diagnosis and treatment of different types of cancers and central nervous system diseases [14, 75, 80, 83]. For example, it is possible to target TfR by transferrin conjugates through the use of MAbs or their fragments that are specific for TfR1 and potentially specific for TfR2 [27, 78].

Targeting the TfR in this way has been shown to be effective in delivering therapeutic agents specifically into tumor cells and causing cytotoxic effects including growth inhibition and/or induction of apoptosis in a variety of malignancies *in vitro* and *in vivo*. For instance, the chimeric anti-TfR IgG3-Av antibody fusion protein developed by *Tracy R et al.*, is a unique molecule that exhibits both intrinsic cytotoxic activity with the ability to deliver a wide variety of biotinylated therapeutic agents into cancer cells.

More advances in this area are expected to further improve the therapeutic potential of targeting the TfR [78, 88]. Generally, targeting cancer cells through use of the TfR can enhance drug delivery by increasing intracellular drug concentration resulting in more effective tumor targeting, less non-specific toxicity and therefore in an overall increased therapeutic efficacy [27].

Peptides as Anticancer Drug Targeting Carriers: Although the sustained prevalence of cancer continues to motivate a dramatic acceleration in the discovery and development of new and highly potent therapeutic molecules, many have not achieved clinical use due to poor delivery, low bioavailability and/or lack of specific targeting [3, 89].

Fortunately, significant research efforts have been directed towards targeting cancer drugs to tumors using specialized drug carriers, and peptides have become an important component of these targeting approaches [3, 90].

Peptide-based targeting of tumor-associated receptors is an attractive approach in tumor-specific drug delivery because the recent advances in phage display technology, combinatorial peptide chemistry and biology have led to the identification of a richly varied library of bioactive peptide ligands and substrates, and the development of robust strategies for the design and synthesis of high-affinity peptide sequences that can be used as drugs and biological tools [50, 89].

Concurrently, with booming research in nanotechnology for biomedical applications, peptides have been studied as an important class of components in nanomedicine, and they have been used either alone or in combination with other nanomaterials in cancer nanomedicine, as drug carriers, as targeting ligands and as protease-responsive substrates for drug delivery [3, 89, 91]. As nanomaterials, peptides possess many advantages: they are relatively small, can be easily synthesized and modified by chemical methods on a large scale, can be facilely conjugated to other molecules, and have good biocompatibility and low generic cytotoxicity.

In addition, advances in peptide manufacturing have reduced the cost of manufacturing peptides and have enabled small companies to participate in the development of peptides that can be used as therapeutic drugs, the substrates of disease site-specific stimuli, such as protease and/or structural components of nano-sized carriers that can bind to specific targets with high affinity and can facilitate cellular delivery of cargoes such as cytotoxic drugs and imaging agents [3, 89, 90].

Some of those peptides that have promising potential in targeting anticancer drugs and imaging agents are briefly discussed as follows;

1. **Tumor targeting by Stable Toxin Peptides:** The human *E.coli* heat-stable enterotoxin (STh) is a 19-amino acid peptide that specifically targets the guanylate cyclase C receptor (GCC). GCC is a type I transmembrane glycoprotein that is present in high density on the apical surface of normal intestinal epithelial cells as well as highly expressed on the surface of human colorectal cancer cells. Several studies have established the applicability of radio-labeled STh such as ^{111}In-labeled STh analogs to the diagnosis of human colorectal cancers *in vivo.* *Gibli MF et al.,* had described the use of ^{90}Y- and ^{177}Lu -labeled STh analogs for peptide receptor radiotherapy [15].

   In addition, different other analogs of STh are currently being used as vectors to target human colon cancers [15, 92]. For example, STh that is genetically fused to another heterologous protein still retains its native biological properties such as secretion, enterotoxicity, folding, GCC recognition and which is poorly antigenic, or almost non-immunogenic has been established a highly attractive tool as a cell targeting and delivering vector, not only for small therapeutic molecules like radionuclides, but also for large proteinaceous anticancer agents [93].

2. **Cell penetrating peptides for Targeting Anticancer Dugs:** The cellular plasma membrane possesses an effective barrier for most hydrophilic macromolecules. The need to deliver biologically active agents into cells has encouraged researchers to develop various delivery vectors. Unfortunately, most delivery systems suffer from different limitations that need to be overcome to be applicable *in vivo* [94].

   In the last two decades, a new class of highly cationic peptides with low molecular weight and with membrane translocation ability was discovered [94, 95]. These peptides were named alternatively as protein transduction domains, cell-penetrating peptides (CPPs) or Trojan horse peptides [10, 95, 96].

   CPPs are heterogeneous in size (10–30 amino acids in length), secondary structure and sequence, and [97, 98] differ considerably in their origin and in their physico-chemical properties [95, 99]. However, they possess multiple positive charges at physiological pH as they are rich in basic cationic amino acids such as arginine, lysine, histidine or proline [97, 100] and some of them share common features, including important theoretical hydrophobicity and helical moment (reflecting the peptide amphipathicity), the ability to interact with lipid membranes and to adopt a significant secondary structure on binding to lipids [97].

   The number of CPPs that have been derived from natural protein and/or designed as totally artificial peptides or chimeras of natural CPPs has increased to about 100 within the last two decades [99, 101]. HIV-1 Tat peptide (or pTat (48–60)) is the first CPP that was isolated from the HIV transcription activating factor in 1988 [95, 98, 102]. Penetratin™(also named pAntp (43-58)) , which is a sequence of 16 amino acids from the third helix of the Drosophila melanogaster antennapedia transcription factor homeodomain protein (amino acids 43–58), is the other commonly used CPP that was derived from naturally occurring non-viral proteins in 1991 [94, 95, 98, 101].

   Both of these CPPs contain a high density of basic amino acids such as arginines and/or lysines, which are proposed to interact with the anionic surface of the plasma membrane and enhance internalization of the peptides [101, 103]. Since these initial observations, multiple CPPs have been discovered from natural origins and chimeric, synthetic peptide sequences have been also

derived, including transportan, HSV-1 protein VP22 and MPG, model amphipathic peptide (MAP), oligoarginines (R7 or R9) and polyarginine [94, 95, 102, 104, 105]. Several of the CPPs identified so far are 9-35mer cationic and/or amphipathic peptides that have the ability to cross the lipid layer barrier of the plasmatic membrane of several cells, usually impermeable for biological molecules [95, 106].

This led to the recognition of CPPs as effective and non-toxic mechanism to mediate the translocation of different conjugated cargoes (e.g., anti-cancer therapeutics) across the plasma membrane of target cells [96]. The vehicular potential of CPPs was realized in 1995 when studies on pAntp demonstrated that the peptide could be attached to a bioactive compound (forming a 'conjugate') and used to achieve its intracellular delivery. Significantly, the attached cargo (a protein kinase C inhibitor) retained its function upon internalization into a live neuron [98].

Importantly, the *in vivo* potential for CPPs to act as vectors for therapeutically active macromolecules was realized in 1999 when Schwarze *et al.* described the delivery of a 120-kDa recombinant β-galactosidase protein fused to the TAT domain to the cytoplasm of several tissues [98, 102]. Following this achievement, several studies had been conducted in the last one decade to use different CPPs for delivery of many biologically active compounds, including various large molecules in to cells so that they can exert their therapeutic action inside cytoplasm or onto nucleus or other specific organelles, such as mitochondria [103, 106].

Several studies by different research groups had shown that CPPs can be used for the delivery of a wide range of molecular cargoes, including imaging agents (fluorescent dyes and quantum dots), drugs, proteins, peptides, antibodies, toxins, DNA, antisense oligonucleotides, siRNAs, HPMA polymers, liposomes, nanoparticles, bacterio-phages, adenovirus, plasmid DNA, phosphorothioate oligonucleotides, peptide nucleic acids, streptavidine, paramagnetically labeled DOTA, several fusion proteins/peptides and iron beads into cells [96, 102, 104, 107, 108].

One of the highlighting features of CPPs as useful tools for intracellular delivery of therapeutic macromolecules is the huge diversity of the transported molecules in terms of size and biological nature[108]. Moreover, when they are covalently linked to larger and poorly internalized macromolecular cargoes such as proteins, polypeptides and nucleic acids, they still retain their translocation properties. In addition, these peptide-based vectors are considered as biocompatible and economical candidates for delivery of hydrophilic drugs [107, 109].

Even though CPPs have been successfully applied, their mechanism of cell entry is not completely elucidated. Increasing evidence indicates that there may be several different pathways involved, depending on the properties of the CPP, attached cargoes, concentration and cell type [94, 96, 110]. Among several mechanisms by which CPPs may mediate intracellular cargo delivery, several have suggested that the endocytic pathways as the primary routes of uptake for various CPPs. According to this mechanism, CPPs, particularly those with a high content in cationic residues, are first simply adsorbed at the cell surface thanks to the numerous anionic moieties, such as heparan sulfate proteoglycans, sialic or phospholipidic acid. Then CPP-mediated transport has been reported to happen through different endocytosis routes: via caveolae, macropinocytosis, through a clathrin-dependent pathway, via a cholesterol-dependent clathrin-mediated pathway or in the trans-Golgi network [105, 111, 112].

The identification of CPPs as vectors for the intracellular delivery of different conjugated molecular cargoes has several advantages over conventional techniques because it is efficient for a range of cell types, can be applied to cells en masse, and has a potential as a targeting strategy for therapeutic applications [103, 106]. A few anti-cancer or cytotoxic drugs have successfully been delivered by CPPs; the anti-cancer drug doxorubicin has been delivered by using the CPPs Penetratin, synB3 and pTat and methotrexate has been delivered by using the newly designed CPPs, YTA2 and YTA4 to minimize toxicity and to battle drug resistance [110].

Furthermore, Aroui S *et al,* had demonstrated that an unfolded analogue of the maurocalcine peptide (MCaAbu) acts as a potent vector for the intracellular delivery of doxorubicin into two models of breast cancer cell lines MDA-MB 231, relatively doxorubicin-resistant, and MCF7, doxorubicin-sensitive, respectively.

It had been validated that the doxorubicin–MCaAbu conjugate is as effective as doxorubicin–Tat and doxorubicin–Penetratin conjugates for overcoming reduced doxorubicin sensitivity in MDA-MB 231 compared to MCF7 cells [113]. In addition, CPPs that are tumor targeting peptides have been developed as promising vehicles for site-directed cancer therapy. For example, Pep42, a cyclic 13-mer oligopeptide that specifically binds to glucose-regulated protein 78 (GRP78) and internalized into cancer cells, represents an excellent vehicle for tumor cell-specific chemotherapy [114].

Various other peptides with specific binding activity for a given cell line (cell-targeting peptides) have also been reported in the literature [112]. One of the goals of the next years will be to optimize the tissue and cell delivery of therapeutic molecules by means of CPPs which combine both targeting and internalization advantages.

SUMMARY: To date, cancer remains one of the world's most devastating diseases. Chemotherapy is still one of the most effective approaches to cancer treatment. However, the crucial problem in cancer chemotherapy is the adverse toxic side effects of anticancer drugs on normal tissues and cells due to limited selectivity of most common drugs for target tumor cells. To limit the severe side effects of cancer chemotherapy on healthy cells, tissues or organs, the tumor-targeting drug delivery system needs to be developed.

So far selectively targeting a tumor cell population utilizing the passive and/or active targeting approach has undergone considerable development over the last decades. Passive targeting of nanoparticles to tumors occurs by the modulated vasculature, which allows nanocarriers to extravasate through gaps in the endothelium.

The entry of the particles to the interstitial space, associated with poor lymphatic drainage from the tumor, results in higher retention times of nanoparticles in the tumor than in normal tissues, in a process known as the EPR effect. Significant increases in drug accumulation in the tumor tissue by the EPR effect can reach 10- fold or higher concentration with drug-loaded nanoparticles compared to free drug.

Active targeting on the other hand is delivering drugs to a specific tumor tissues in terms of molecular recognition with a suitable ligand which can recognize its receptor on the targeting tumor cells. Among all the neoplastic targeting ligands that are presently under use and/or investigation, the different protein-based ligands such as MAbs, BsAbs, Affibody molecules, albumin, transferrin, and peptide-based ligands such as microbial toxins and CPPs are the popular ones used in targeting anticancer payloads to specific tumor cells.

In general active targeting is one of the most promising strategies to emerge that involves the conjugation of a cytotoxin payload to a tumor targeting protein/peptide through an acid-labile linker that is stable at physiological pH.

Internalization at the target tumor site via processes such as receptor mediated endocytosis exposes the conjugate to the acidic environment of the endosomes or lysosomes, resulting in selective release of the cytotoxin payload inside the tumor cell and localized cell death. Attaching a cytotoxin payload to a tumor targeting protein/peptide through a selectively labile linker not only reduces its general toxicity to normal tissues, but can also significantly improve the pharmacological properties of the cytotoxin agents.

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