The Potential and Current Progress of Internalizing Molecules in Targeted Drug Delivery

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Abstract Safe and efficient drug delivery in the treatment of cancer and infectious diseases constitute a major challenge. Development of targeted intracellular delivery approaches to specific cell populations or tissues for different therapeutics is highly desirable to maximize potency and minimize toxicity. Extensive efforts to improve drug safety and effectiveness have resulted in numerous target-specific delivery strategies. Currently, various internalizing molecules, including antibodies, proteins, peptides, folate, carbohydrates, aptamers and other ligands, have been successfully adopted as active recognition moieties to target a distinct disease or tissue in a cell-type-specific manner. The use of direct covalent conjugation or non-covalent assembly of targeting ligands with the drug or delivery vehicle can be used to get drugs to their intended targets, thereby reducing the dosing requirements as well as minimizing unwanted toxicities. In this article we focus mainly on the potential and current progress of internalizing molecules in targeted drug delivery.

Keywords Cell-type specific delivery • Internalizing molecules • Targeted drug delivery • Targeted therapy

Abbreviations

AML	acute myeloid leukemia
Antp	antennapedia
Apo A-I	apolipoprotein A-I

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Ara-C	1-β-D-arabinofuranosylcytosine
AZT	azidothymidine
CCP	cell-penetrating peptide
CD4	cluster of differentiation 4
CD7	cluster of differentiation 7
CPT	camptothecin
DRBD	double stranded RNA-binding domain
CVB3	coxsackievirus B3
d4T	stavudine
Dox	doxorubicin
Dtxl	docetaxel
ECM	extracellular matrix
EFV	efavirenz
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPR	enhanced permeability and retention
HDL	high-density lipoprotein
GnRH	gonadotropin-releasing hormone
HA	hyaluronic acid
HBV	hepatitis B virus
HCV	hepatitis C virus
HER-2	human epidermal growth factor receptor-2
HIV gp120	HIV glycoprotein 120
LHRH	luteinizing-hormone-releasing hormone
LN	lymph nodes
mAb	monoclonal antibody
MAP	mitogen-activated protein
MR	magnetic resonance
MTX	methotrexate
MUC1	mucin protein
NCL	nucleolin
NP	nanoparticles
NR	nanorod
NRP1	neuropilin-1
ON	oligonucleotide
PAMAM	polyamidoamine
PEI	polyethylenimine
PHA	polyhydroxyalkanoates
PL	phospholipid
PLL	poly-L-lysine
PPI	poly(propyleneimine)
pRNA	packaging RNA
PSMA	prostate-specific membrane antigen
PTD	peptide transduction domain
PTK7	protein tyrosine kinase 7
QD	quantum dot

RNAi	RNA interference
scFv	single-chain Fv antibody
SELEX	systematic evolution of ligands by exponential enrichment
siRNA	small interfering RNA
SR-BI	scavenger receptor BI
sTRAIL	tumor necrosis factor-related apoptosis-inducing ligand
SWNT	single-wall carbon nanotube
TAT	trans-activating transcriptional activator
3TC	lamuivudine
Tf	transferrin
TfR	transferrin receptor
TN-C	tenasin-C
TNPO3	Transportin-3

1 Introduction

Since the "magic bullet" dream was first postulated by Dr. Paul Ehrlich in 1906 (Strebhardt and Ullrich 2008), "targeted therapy" has become a long-standing goal for human disease treatment but still remains a major challenge for clinical applications (Zhukov and Tjulandin 2008; Markman 2008; Katzel et al. 2009). Most therapeutic agents currently in use, such as conventional chemotherapy (Joensuu 2008), radiotherapy (Harrison et al. 2002), immunotherapy (Shapira et al. 2010) or gene therapy (Whitehead et al. 2009), generally are not functionalized to selectively target the site of disease. When these therapeutics agents are systemically administrated, they nonspecifically distribute throughout the body, thereby significantly reducing the therapeutic efficacy and leading to harmful side-effects associated with distribution to non-targeted sites (Langer 1998).

With the intent and enthusiasm for developing targeted therapeutic drug delivery strategies, many efforts have been made to develop targeted drug delivery strategies capable of selectively transporting drug to a specific site of disease (Yu et al. 2010; Zhou and Rossi 2009; Kim et al. 2009c; Levy-Nissenbaum et al. 2008; Allen 2002). The strategy mainly relies on specific interaction between the targeting ligand and its receptors expressed on the diseased cells or tissues. Such ligand-directed recognization events consequently increase the local concentration of the drug in the targeted cells or tissues. Furthermore, after the ligand-receptor binding, the cellular receptors are readily internalized and rapidly re-expressed on the cell surface to allow repeated targeting and internalization. Depending on the types of therapeutic agents or drug formulation, it is worth noting that internalization may be dispensable in some therapies (Allen 2002). For example, for antibody-directed enzyme-prodrug anticancer therapy (Senter and Springer 2001), the enzyme-prodrug is first selectively accumulated on the cell surface *via* antibody-receptor interaction; subsequently, it is activated into an anticancer drug by the enzyme that only functionalizes at the cell surface. Additionally, in the radioimmunotherapy (Ercan and Caglar 2000; Goldenberg 2002), antibody-conjugated beta- or alpha- radionuclides can directly

functionalize at the cell surface once the conjugates are selectively localized on the target cells or tissues. However, internalization might still be beneficial for some alpha-emitting radionuclides with a very short range of alpha-particle emission and lack of penetrability. In this regard, an internalizing ligand therefore might more be efficient and helpful than non-internalizing ligands. Indeed, a specific internalizing molecule with high specificity and affinity to a cellular surface receptor is therefore a major factor in establishing a targeted drug delivery system.

Over the past few decades, a wide variety of internalizing molecules such as antibodies, proteins, peptides, folate, carbohydrates, aptamers and other small molecule ligands have been successfully adapted for the targeted delivery of active drug substances both *in vitro* as well as *in vivo* (Russ and Wagner 2007; Ciavarella et al. 2010; Yan and Levy 2009; Higuchi et al. 2010). Recent investigations have described targeted delivery of various therapeutic agents (e.g. anticancer, antifungal, antiviral agents, antibiotics, protein, peptide, genes, oligonucleotides and small interfering RNAs) using different strategies. Through precisely engineering the internalizing molecules with the drug or delivery vehicle, the recognition and internalization of the therapeutic compounds by the target tissue can be dramatically improved. In particular, the advent of nanotechnology has greatly accelerated the development of drug delivery, providing a large variety of nanocarriers for disease therapy including liposomes, polymers, carbon nanotubes and quantum dots etc. (Mok and Park 2009; Gullotti and Yeo 2009; Kim et al. 2009a; Wiradharma et al. 2009; Liu et al. 2007b; Zrazhevskiy and Gao 2009). Meanwhile, powerful multifunctional nanomedicines that combine several desirable functions such as therapeutics, targeting and imaging in one nanoscale carrier, have been developed to significantly enhance drug efficacy (Torchilin 2006; Zrazhevskiy and Gao 2009; Gao et al. 2010; Muthu and Wilson 2010; Hart 2010). In this review, we discuss recent advances in targeting strategies for tumors and infectious diseases, with a particular emphasis on cell internalizing molecules as a targeting strategy.

2 Targeting Strategies for Targeted Delivery

The key for targeted therapy is to accurately identify molecular targets that distinguish diseased cells from healthy ones. Ideally, a targeted drug delivery should enable selective accumulation of the drug in the specific target cells with minimal adverse side-effects. Generally, targeted delivery can be realized through two strategies (Torchilin 2010): (**1**) passive targeting and (**2**) active targeting.

2.1 Passive Targeting

In the first strategy, macromolecules and nanocarriers with sizes below 400 nm in diameter can travel through the bloodstream and accumulate in tumor *via* passive

leakage, thereby increasing the concentration of drugs in tumors and enhancing the therapeutic index (Yuan et al. 1995; Moghimi et al. 2001). This passive mechanism is called the enhanced permeability and retention (EPR) effect (Matsumura and Maeda 1986; Greish 2007). Since passive targeting relies on a size-flow-organ/tissue filtration that is generally limited to tumors and lymph nodes (LNs), drug particles/carriers can theoretically be designed and engineered with appropriate sizes (e.g. 100–400 nm) and surfaces (e.g. PEGylated surface) (Maeda 2010). Currently, many studies have taken advantage of the EPR effect to achieve passive targeting of drug nanocarriers to most human tumors. And some of them have been approved for clinical use such as Doxil (Doxorubicin encapsulated by PEGylated liposome) (Gabizon 2001). In the treatment of HIV, LNs are important sites for targeting HIV-1 replication and they have been exploited as a promising passive targeting site (Gupta and Jain 2010; Gunaseelan et al. 2010).

2.2 Active Targeting

In the second strategy, an actively recognized moiety specific to the target sites of interest is an essential component. These actively recognized molecules can be precisely decorated on the therapeutic agents or their delivery vehicles, functioning as cell or tissue-specific homing agents (Peer et al. 2007; Gullotti and Yeo 2009). Typically, by utilizing biologically specific interactions such as antigen-antibody binding or ligand-receptor interactions, these targeting molecules facilitate the cellular uptake of therapeutic agents *via* receptor-mediated endocytosis or cellular membrane permeation, thereby increasing the local concentration of the drug in the targeted cells or tissues, thereby improving the therapeutic efficacy at lower doses.

2.3 Combinatorial Targeting

Despite numerous successful examples using either one of the above strategies, a combination of passive and active targeting also has been exploited to provide the most efficient targeted delivery system (Torchilin 2010). In this regard, a combinatorial targeting results in twice the enrichment at the cellular and tissue/organ levels (Akbulut et al. 2009). For example, for a targeted nanocarrier system, an appropriate nano-scale size can allow preferential accumulation in the tumor tissue/organ in the passive mode. Once the nanocarrier is concentrated at the tumor site, a cell type-specific affinity internalizing molecule such as an antibody, peptide, folate or aptamer engineered on the nanocarrier surface would further facilitate selective internalization into the targeted tumor cells. In comparison with traditional small molecule drugs or non-targeted nanocarriers, targeted nanocarriers that combine the above two desirable properties could not only prolong circulation time and

improve drug biodistribution *via* passive targeting, but would also provide higher specificity *via* the active targeting component (Park et al. 2009).

3 Specific Internalizing Molecules for Targeted Delivery

Currently, the development of the internalizing molecules specifically targeting membrane receptors and their adaptation for active drug targeting has drawn attention in the field of targeted therapy. In the following section internalizing molecule-mediated active targeting will be discussed in greater detail.

3.1 Antibody-Mediated Drug Delivery

Antibodies (Abs, also known as immunoglobulins) that are used by the immune system to identify and neutralize foreign objects can specifically identify and bind their unique antigens. Due to the specificity of antigen-antibody binding, different internalizing antibodies and their genetically engineered fragments have been widely investigated as active targeting molecules (Wu and Senter 2005; Schrama et al. 2006). These include anti-CD33 monoclonal antibodies (mAb) (Simard and Leroux 2010), anti-HER2 mAb (anti-CD90 mAb) (Park et al. 2002; Chiu et al. 2004a), anti-CD7 mAb (Kumar et al. 2008), anti-HIV envelop gp120 mAb (Song et al. 2005), anti-β7 integrin mAb (Peer et al. 2008), anti-JL1 mAb (Suh et al. 2001), anti-EGF receptor Ab (Wu et al. 2004; Mamot et al. 2005), anti-CD31 (Li et al. 2000), anti-GAD Ab (Jeong et al. 2005), anti-D4.2 mAb (Ho et al. 1987), anti-H-2K^k mAb (Connor and Huang 1986), anti-HLA-DR Ab (Gagne et al. 2002), anti-DC-SIGN mAb (Tacken et al. 2005; Dakappagari et al. 2006) and so on. Four representative antibodies targeting cancer cells or HIV infected cells are described below.

3.1.1 Anti-CD33 Antibody

The CD33 receptor is a 67 kDa glycoprotein expressed on the surface of leukemia cells in most patients with acute myeloid leukemia (AML) (Griffin et al. 1984; Scheinberg et al. 1989). Previous studies have demonstrated that CD33 can be rapidly internalized into leukemia cells after antibody binding (Simard and Leroux 2009). Therefore, anti-CD33 mAbs have been conjugated with cytotoxic agents or nanocarriers for targeted delivery. For example, Mylotag (Gemtuzumab Ozogamicin), a recombinant, humanized anti-CD33 antibody which was approved by the US Food and Drug Administration in 2000 as a single-agent therapy for CD33-positive AML, was linked with an anticancer agent (calicheamicin) and the resulting conjugate showed excellent clinical promise (Larson et al. 2005). Recently, an anti-CD33

mAb or its Fab' fragment were also chemically coupled to PEGylated liposomes (LPs) (Simard and Leroux 2010). Such a liposomal formulation has been demonstrated to specifically recognize the CD33 cell surface antigen and promote the efficient intracellular delivery of 1-β-D-arabinofuranosylcytosine (ara-C, anti-AML agent) to human myeloid leukemia cells.

3.1.2 Anti-HER2 Antibody

Another popular antibody for tumor targeting is the anti-HER-2 (human epidermal growth factor receptor-2) antibody (Mamot et al. 2005; Wu et al. 2004). Since HER-2 is highly-expressed on the surface of many human pancreatic cancer cell lines, it can be used as a therapeutic target. Therapeutic antibodies such as Trastuzumab and Herceptin have been routinely applied in the clinical treatment of breast cancer and leukemia (Chiu et al. 2004a). A number of reports have shown that the anti-HER2 antibody can be decorated on a nano-particle surface *via* avidin-biotin or covalently conjugated to polymers (e.g. polyethylenimine PEI) through different crosslinkers. Moreover, anti-HER2 Ab-mediated delivery of oligonucleotides encased in lipid nanoparticles was also shown to be capable of targeting mammary carcinoma cells (Kirpotin et al. 2006). These modified nanocarriers were effectively endocytosed by HER2-overexpressing cells. In addition, a multifunctional anti-HER2 Ab conjugated magnetic gold nano-shell particle showed the potential for targeted MR (magnetic resonance) photo-thermal therapy and tumor imaging (Kim et al. 2006).

3.1.3 Anti-CD7 Antibody

CD7 (Cluster of Differentiation 7), a human transmembrane protein, is found on thymocytes and the majority of human T cells. It has been documented that CD7 is rapidly internalized after antibody binding. Several research groups have exploited an anti-CD7 mAb and its recombinant single-chain Fv (scFvCD7) fragment for targeted delivery. For example, a single-chain Fv fragment was genetically linked to a truncated Pseudomonas exotoxin A fragment, thereby conferring restricted specificity for CD7 positive cells (Peipp et al. 2002). The recombinant immunotoxin promoted apoptosis in two CD7-positive cell lines and provided a potent inducer of apoptosis in acute leukemic T cells. In a similar manner, scFvCD7 was genetically fused with sTRAIL (tumor necrosis factor-related apoptosis-inducing ligand) (Bremer et al. 2005). As expected, treatment with scFvCD7 : sTRAIL specifically induced potent CD7-restricted apoptosis in a series of malignant T-cell lines, with low toxicity for normal human blood and endothelial cells.

In addition to the targeted delivery of a toxin, the scFvCD7 Ab was recently used for siRNA delivery *in vitro* and *in vivo*. Kumar et al. conjugated a Cys-modified scFvCD7 with an oligo-9-arginine peptide to obtain a cell type specific siRNA delivery system (scFvCD7-9R) (Kumar et al. 2008). A combination of siRNAs

against the cellular CCR5 co-receptor and two conserved HIV-1 genes (*vif* and *tat*) was loaded into scFvCD7-9R system, subsequently was systemically administered to HIV-1 infected animal model. The weekly injection resulted in suppression of HIV-1 infection and protection of CD4+ T cell depletion in humanized mice.

3.1.4 Anti-HIV gp120 Antibody

The interaction of the HIV envelope glycoprotein gp120 with the cellular CD4 receptor is a crucial step in the entry of HIV into T-cells (Ugolini et al. 1999; Sattentau and Moore 1993). HIV gp120 is exposed on the surface of virus particles and the plasma membrane of HIV-1 infected cells. Therefore, it represents an excellent marker to distinguish HIV-1 infected host cells from non-infected cells (Kwong et al. 1998). F105, an anti-HIV gp120 monoclonal antibody, has been demonstrated to bind with high affinity to its ligand gp120 expressed on the surface of a wide range of HIV-1 laboratory strains and primary isolates (Posner et al. 1991). Importantly, its selective binding to HIV-1 infected cells triggers rapid cellular internalization. Thus, F105 has the potential to be an excellent internalizing molecule for selective drug delivery to HIV-1 infected cells (Clayton et al. 2007). Song et al. have successfully applied a heavy chain fragment of F105 to increase receptor-specific uptake to cells expressing the HIV envelope protein (Song et al. 2005). In this system, the F105 antibody fragment – protamine fusion protein was able to specifically bind to and deliver siRNAs to HIV-infected primary T cells or HIV envelope-expressing cells. Moreover, their results demonstrated that the siRNA targeting the HIV-1 *gag* capsid gene delivered by such a fusion protein inhibited HIV-1 replication only in cells expressing the HIV-1 envelope. Recently, PEGylated liposomes coated with a F105 Fab' fragment have been developed and evaluated for targeted delivery of a novel HIV-1 protease inhibitor PI1 (Clayton et al. 2009). The anti-HIV immunoliposomes were selectively taken up by HIV-1 infected cells. When compared with free drug or non-targeted drug, the targeted PI1 delivery showed a greater inhibitory effect on viral replication.

3.2 Protein-Mediated Drug Delivery

3.2.1 Transferrin and Lactoferrin

Transferrin (Tf), an 80 kDa glycoprotein that transports iron into cells, is one of most popular tumor targeting ligands, because its receptors (TfRs) are over-expressed in many types of cancer cells and TfR expression is correlated with the proliferation and malignancy of the tumor cells (Daniels et al. 2006). After interaction of Tf-TfR, Tf is internalized into cells through an endocytosis pathway (Qian et al. 2002). To date, numerous investigations have demonstrated that Tf-conjugated nanocarriers loaded with various drugs (e.g. anticancer agents, oligonucleotides, therapeutic genes, siRNAs) could facilitate specific cellular uptake and enhance the

therapeutic efficacy of the drugs in tumor cells (Heidel et al. 2007; Davis 2009). One group developed a Tf-conjugated cyclodextrin-containing polycation delivery system for siRNAs, which resulted in 50% greater gene silencing in the tumor versus a non-targeted delivery system in an immunodeficient mouse model (Bartlett et al. 2007). Most recently, the same group conducted the first in-human phase I clinical trial using a Tf-targeted nanoparticle delivery system and their work provided direct evidence of intercellular delivery of the siRNA which triggered the RNA interference mechanism (Davis et al. 2010).

Since the brain capillaries are specifically known to express TfR, Tf-conjugated nanocarriers were also successfully applied for brain targeting (Chang et al. 2009). For example, Tf chemically conjugated to polyamidoamine (PAMAM) dendrimers resulted in an increase in the brain uptake of the therapeutic DNA (Huang et al. 2007). In a similar study, Tf-coated PEG nanoparticles delivered an antiretroviral agent (azidothymidine, AZT) across the blood-brain barrier to the central nervous system (Mishra et al. 2006). Additionally, another glycoprotein lactoferrin, a globular iron-binding protein, also could function as a lung and brain targeting molecule (Elfinger et al. 2007). It is known that lactoferrin receptors are predominantly expressed in brain capillaries and bronchial epithelial cells, thereby suggesting a new target for brain delivery as well (Huang et al. 2008a).

3.2.2 Epidermal Growth Factor (EGF)

The epidermal growth factor receptor (EGFR), the cell-surface receptor for members of the EGF-family of extracellular protein ligands, is over-expressed in the majority of human cancers (Muslimov 2008). EGF acts by high affinity binding to EGFR on the cell surface thereby stimulating the intrinsic protein-tyrosine kinase activity of the receptor (Agarwal et al. 2008). Through the formation of dimers EGF is endocytosed into the cells after receptor-binding. Moreover, because EGF only has 53 amino acids and a molecular weight of ~6 kDa, it has been widely employed as an internalizing ligand for tumor targeting. A number of such examples have been reported in the literatures (Lee et al. 2003; Lee and Park 2002). For example, biotin-modified EGF molecules were installed onto a Streptavidin-modified PEI complexed plasmid DNA *via* the interaction of biotin-Streptavidin (Lee et al. 2002). The resulting EGF-targeted PEI delivery system resulted in a significantly higher transfection efficiency than non-targeted system in a human epidermal carcinoma cell line. Recently, EGF was attached to single-wall carbon nanotubes (SWNTs) loaded with cisplatin or quantum dots (QDs) and used for targeted therapy and tumor imaging (Bhirde et al. 2009).

3.2.3 Apolipoprotein A-I

Apolipoprotein A-I (apo A-I) is a protein component of the high-density lipoprotein (HDL) (von Eckardstein et al. 2001). It has been demonstrated that apo A-I can predominantly be internalized to the liver *via* the cell-surface receptor scavenger

receptor BI (SR-BI) (Kim et al. 2007). As a typical serum protein, apo A-I functions as a beneficial factor to decrease cholesterol levels and therefore should not trigger immunological side effects in clinical applications. Thus, apo A-I can be used as an internalizing moiety for liver targeting. A proof-of-concept study has shown the potential of apo A-I for the systemic delivery of nucleic acids to the liver using real-time *in vivo* imaging (Kim et al. 2007). Apo A-I was formulated onto the surface of the lipid bilayer of a representative cationic liposome and facilitated the hepatocyte-specific siRNA delivery *via* receptor-mediated endocytosis. The Apo A-I-coating liposome carrying an anti-hepatitis B virus (HBV) siRNA showed a prolonged inhibition of the target protein expression at low doses in a mouse model. A subsequent study further evaluated the efficacy of an apo A-I – mediated liver-specific delivery of an siRNA against hepatitis C virus (HCV) in a transient HCV model (Kim et al. 2009b). The results of this study showed Apo-liposome nanoparticles systemically delivered an siRNA into mouse hepatocytes expressing HCV resulting in siRNA mediated inhibition of the targeted HCV protein expression.

3.3 Peptide-Mediated Drug Delivery

3.3.1 RGD Peptide

Peptides based on the three-amino-acid sequence arginine-glycine-aspartate, known as RGD peptides have been used extensively as tumor cell-recognizable ligands in tumor diagnostics and therapeutics (Ruoslahti 2003). The RGD sequence, originally identified as a cell binding site in the extracellular matrix (ECM) protein (fibronectin and vitronectin), can bind to the integrin receptor $\alpha_v\beta_3$ with high affinity (Cheresh 1987). Since the integrins are specifically over-expressed at the surface of tumor cells and angiogenic endothelial cells at the tumor site, RDG-mediated drug delivery generally leads to high levels of accumulation in tumor tissues compared with free drug or non-targeted drug delivery systems. Both linear RGD and cyclic RGD have been popularly applied for targeted delivery of traditional drugs, genes and polymers. For example, a dimeric RGD peptide was conjugated to an oligonucleotide (ON), thereby increasing cellular uptake and the biological function of the ON (Alam et al. 2010; Alam et al. 2008). Additionally, Dai et al. chemically conjugated a cyclic RGD peptide to the terminal group in a phospholipid – single-walled carbon nanotube (PL-SWNT), which resulted in enhanced and specific delivery of doxorubicin (Dox) to RGD positive U87 cancer cells (Liu et al. 2007a).

Recently, Ruoslahti and colleagues identified a cyclic peptide iRGD (CRGDKGPDC and its related variants), which can target tumor cells by binding α_v integrins that are specifically expressed in the tumor vessel endothelium (Sugahara et al. 2009). They combined the tumor-homing RGD sequence with another peptide ligand (the CendR motif) for neuropilin-1 (NRP1, a transmembrane receptor) that mediates penetration into tissue and cells. The resulting peptide-mediated delivery system (iRGD) was first recruited to tumor tissue through the

RGD-integrin interaction and subsequently was proteolytically processed to generate a CendR motif with cell-penetrating activities, further facilitating the penetration of drug into the tumor. Therefore, drugs chemically conjugated to the iRGD could be selectively transported deep into the tumor parenchyma, and significantly improve the sensitively of tumor-imaging agents and the activity of an antitumor drug. Recent studies further demonstrated that co-administration of drugs and the iRGD peptide resulted in penetration of extravascular tumor tissue (Sugahara et al. 2010). They demonstrated in murine tumor models that systemic injection with iRGD can improve the therapeutic index of various therapeutic agents, such as a small molecule drugs (doxorubicin), nanoparticles (nab-paclitaxel and doxorubicin liposomes), and a monoclonal antibody (trastuzumab). Although the mechanism of action still requires further investigation, this system may represent a valuable strategy to enhance the efficacy of anticancer agents and to accelerate clinical translation of modification-intolerable drugs.

3.3.2 LHRH Peptide

Luteinizing-hormone-releasing hormone (LHRH), also known as luliberin and Gonadotropin-releasing hormone (GnRH), are known to be internalized and are good agents for tumor targeting (Dharap et al. 2003, 2005; Kim et al. 2008). Although LHRH receptors are present on the surface of most healthy human cells, they are over-expressed in ovarian, prostate and breast cancer cells. Through direct conjugation, the LHRH peptide or its analogues have been applied for the delivery of anticancer drugs and siRNAs. For example, Dharap et al. have shown that a LHRH-PEG-camptothecin (CPT), a cytotoxic quinoline alkaloid which inhibits the DNA enzyme topoisomerase I, significantly enhanced the drugs cytotoxicity against cancer cells (Dharap et al. 2003). Recently, a LHRH peptide analogue was appended to an siRNA *via* a PEG linker to target ovarian cancer cells (Kim et al. 2008). Once effective cellular internalization of the LHRH-targeted siRNA conjugates takes place the disulfide bond between PEG and siRNA is reductively cleaved to release the siRNA into the cytosol.

3.3.3 Peptide Transduction Domains (PTDs)

PTDs (also called cell-penetrating peptides), are short cationic peptide sequences with a maximum of 30 amino acids that mediate translocation across cell membranes (El-Sayed et al. 2009; Pangburn et al. 2009). Despite the different theories on the mechanism of PTD-mediated uptake, it is now widely accepted that PTDs bind the anionic cell surface through electrostatic interaction and then are rapidly internalized into cells by macropinocytosis, a specialized type of fluid phase endocytosis that all cells appear to perform (Nakase et al. 2004; Wadia et al. 2004). This feature therefore enhances the cellular uptake in a non-cytotoxic manner, making PTDs attractive candidates for intracellular drug delivery. PTDs such as trans-activating transcriptional

activator (TAT), polyarginine, antennapedia (Antp), penetratin, transportan, and mitogen-activated protein (MAP) have been shown to deliver a wide variety of therapeutic loads to target cells, and some of the most well characterized PTDs are currently being tested in preclinical and clinical trials (Gump and Dowdy 2007).

The most commonly used PTDs is the HIV TAT peptide, a small polypeptide of 86 amino acids with a cysteine-rich region derived from the HIV TAT protein (Rao et al. 2009). This peptide possesses an arginine-rich sequence and is highly positively charged enable permeation of the cell membrane in a receptor- and transporter-independent fashion (Vives et al. 1997). Strong experimental results have validated the effectiveness of TAT-directed drug delivery. The TAT peptide directly conjugated with various agents, including horseradish peroxidase, β-galactosidase and nucleic acids, or decorated on the surfaces of nanocarriers such as liposomes and PEI, is able to deliver these molecules to various cells and tissues in the mouse, displaying high accumulation in the heart, lung and spleen (Pangburn et al. 2009). Chiu et al. covalently linked a TAT peptide to the 3′-terminus of the antisense strand of an siRNA (Chiu et al. 2004b). The Tat-siRNA conjugate showed rapid internalization into cells and specific siRNA mediated knockdown of the target gene.

Despite of these advances in PTD-mediated siRNA delivery, direct conjugation of a cationic PTD to an anionic siRNA often results in insoluble complexes thereby reducing the delivery efficiency and inducing some cytotoxicities (Turner et al. 2007; Meade and Dowdy 2008). In order to overcome this shortcoming, an efficient "mask" PTD-directed siRNA delivery approach that uses a TAT peptide transduction domain-double stranded RNA-binding domain (PTD-DRBD) fusion protein has been developed (Eguchi and Dowdy 2009, 2010; Eguchi et al. 2009). The DRBD is known to bind siRNA with high avidity, which functions as a "mask" to shield the negative charge of the siRNA allowing the PTD to efficiently deliver the siRNA into cells.

3.4 Folate-Mediated Drug Delivery

Folate is also known as folic acid or vitamin B_9, which is able to specifically bind to the folate receptor with nanomolar binding affinity (Weitman et al. 1992; Wang et al. 1996). It has been known that folate receptors are over-expressed in many human cancer cells including ovarian, breast, pharyngeal and liver cancer, while their distribution in normal tissues is minimal. In particular, it was found that expression of folate receptors are elevated on epithelial tumors of various organs such as colon, lung, prostate, ovaries, mammary lands and brain. To date, folate is well-characterized and shows many benefits, including a low-molecular weight (MW 441 Da), good stability, no immunogenicity and well-defined conjugation chemistry. Therefore, it has become one of the most popular targeting moieties for tumor specific drug delivery (Sudimack and Lee 2000). Numerous studies have already demonstrated that attachment of folate to various molecules allows rapid internalization by endocytosis and high accumulation in tumor cells.

Folate has been successfully decorated on polymer, nucleic acids, liposomes, dendrimers, quantum dots and polymeric micelles. For example, a folate-PEG-PEI nanoconjugate delivered plasmid DNA or oligonucleotides into tumor cells in a target specific manner (Cho et al. 2005). Polyhydroxyalkanoates (PHAs) were modified with folate *via* covalent bonds for targeted Dox delivery (Zhang et al. 2010). Similarly, folate-targeted liposomes specifically transported daunorubicin (Ni et al. 2002) and Dox (Goren et al. 2000) into various tumor cells and increased the cytoxic killing of these cells. Folate-linked packaging RNA (pRNA) was shown useful for selectively delivering siRNAs against coxsackievirus B3 (CVB3) thereby effectively inhibiting virus replication (Zhang et al. 2009). In addition, a folate analog, methotrexate (MTX), has been conjugated with magnetic nanoparticles for targeted drug delivery and specific imaging (Duthie 2001).

3.5 Carbohydrate-Mediated Drug Delivery

Lectin-carbohydrate interaction is another consideration for active targeting. It is known that the cell surface is rich in carbohydrate moieties attached to both membrane glycolipids and glycoproteins (Boraston et al. 2004; Kannagi et al. 2004). Therefore, the carbohydrate moieties represent a potential recognition ligand for targeted therapeutic delivery.

3.5.1 Galactose and Lactose

The asialoglycoprotein receptors are expressed on the surface of hepatocytes and are able to mediate endocytosis and subsequent internalization into hepatoma cells. The oligosaccharides, such as galactose (a monosaccharide) and lactose (a disaccharide) have been used as targeting molecules for hepatocyte-specific delivery through asialoglycoprotein receptor-mediated endocytosis (Cook et al. 2005; Lim et al. 2000). For example, a galactosylated liposome was reported to deliver Dox to the hepatocytes (Wang et al. 2006). The attachment of galactose to the cyclodextrin-containing polymer *via* a PEG-adamantine linker allowed for targeted nucleic acid delivery (Bartlett and Davis 2007). Oishi et al. also developed a lactosylated PEG-siRNA conjugate through an acid-labile linkage, which could efficiently release free siRNA once this conjugate entered acidic endosomal compartment (Oishi et al. 2005, 2007).

3.5.2 Mannose

It has been known that mannose receptors are highly expressed in macrophages, dendritic cells and other hematopoietic cells important for innate immune responses. Mannose is recognized by a mannose receptor and is internalized into

cells (Ferkol et al. 1996). The mannose-coating nanoparticles have been widely used for targeted delivery of anti-HIV drugs. For example, Jain et al. have used mannose-conjugated, fifth generation poly(propyleneimine) (MPPI) dendrimers to deliver lamuivudine (3TC) to HIV-1 infected MT-2 cells. Significant increases in the cellular uptake of 3TC were observed compared with free drug and non-targeted PPI dendrimers (Dutta and Jain 2007). In another study, MPPI nanocarriers also delivered efavirenz (EFV) to human monocytes/macrophages. Similarly, stavudine (d4T, brand name Zerit) also has been loaded in mannosylated liposomes and has been evaluated *in vitro* for anti-HIV activity (Dutta et al. 2007).

3.6 Aptamer-Mediated Drug Delivery

Aptamers are evolved, single-stranded nucleic acids that can specifically recognize and bind their cognate targets by means of well-defined stable, three-dimensional structures (Mayer 2009; Famulok et al. 2007). Over the past 20 years, numerous nucleic-acid aptamers have been raised against a wide variety of targets (Nimjee et al. 2005). Moreover, their many favorable characteristics, such as the low nanomolar dissociation constants and exquisite specificity, small size, stability, lack of immunogenicity, and the ability to be chemically synthesized with various base and backbone modifications make them versatile tools for diagnostics, *in vivo* imaging, and targeted therapeutics. Currently, the advances in the development of DNA or RNA aptamers specifically targeting membrane receptors to deliver and enhance the efficacy of other therapeutics agents have drawn attention for exploiting specific cell-internalizing aptamers as targeted drug delivery carriers (Zhou and Rossi 2009; Yan and Levy 2009). An increasing number of cell-type specific RNA and DNA aptamers targeting PSMA (prostate-specific membrane antigen) (Lupold et al. 2002); HIV-1 gp120 (Cohen et al. 2008; Dey et al. 2005); CD4 (cluster of differentiation 4) (Kraus et al. 1998); TN-C (tenasin-C) (Hicke et al. 2001), EGFR (epidermal growth factor receptor) (Li et al. 2010), PTK7 (protein tyrosine kinase 7) (Xiao et al. 2008), TfR (transferrin receptor) (Chen et al. 2008), NCL (nucleolin) (Shieh et al. 2010) and MUC1 (mucin protein) (Ferreira et al. 2009), have been adopted for targeted delivery of the various molecules of interest. Four representative examples are discussed below.

3.6.1 Anti-PSMA RNA Aptamer

PSMA, a trans-membrane protein, is highly expressed in human prostate cancer and the vascular endothelium. It has been known that PSMA can be constitutively endocytosed into PSMA-positive LNCap cells and be continually recycled from the plasma membrane, thereby allowing for intracellular drug transport (Tasch et al. 2001; Liu et al. 1998). Since several 2′-Fluoro modified anti-PSMA RNA aptamers with nanomolar binding affinity were isolated by Lupold et al. (Lupold et al. 2002),

they already have become popular cell-specific internalizing molecules for targeted drug delivery (Zhou and Rossi 2009).

Three independent studies have demonstrated anti-PSMA aptamer-mediated small interfering RNA (siRNA) delivery to PSMA-positive cells or human cancer cells-transplanted into nude mice. Through Streptavidin-biotin interactions, Chu et al. non-covalently assembled two biotinylated anti-PSMA aptamers and two biotinylated siRNAs into a Streptavidin connector (Chu et al. 2006b). The multivalent construct was effectively internalized into targeted cells and triggered specific gene silencing. Using a somewhat different approach, Giangrande and coworkers have developed a simple covalent aptamer-siRNA chimeric RNA which allowed effective PSMA mediated cell uptake along with siRNA-mediated gene silencing in athymic mice following both intra-tumoral delivery and systemic administration (McNamara et al. 2006; Dassie et al. 2009).

By taking advantage of the targeting properties of aptamers, a gelonin toxin has been successfully conjugated to an anti-PSMA aptamer (Chu et al. 2006a). The resulting conjugates dramatically increased cellular uptake and therapeutic efficacy in PSMA-positive cells. Reports from Farokhzad and colleagues also demonstrated the potential of anti-PSMA aptamers to mediate nanoparticles delivery (Peer et al. 2007). For example, they have constructed an anti-PSMA aptamer – poly (lactic acid)-block-PEG copolymer nanoparticle conjugate encapsulating the chemotherapeutic drug Docetaxel (Dtxl-NP-aptamer) (Farokhzad et al. 2004). Such Dtxl-NP-aptamer conjugates showed remarkable efficacy and completely suppressed tumor growth in a xenograft nude mouse model with a single intra-tumoral injection (Farokhzad et al. 2006).

A simple physical conjugation also was employed to assemble an aptamer with drugs. Anthracycline drugs (e.g. Dox) non-covalently intercalated into double-strand regions of an anti-PSMA aptamer *via* the flat aromatic ring, and formed a stable physical conjugate capable of effectively targeting PSMA-positive cells (Bagalkot et al. 2006). In another example, Bagalkot et al. functionalized a quantum dot (QD) with anti-PSMA aptamers to achieve a multifunctional QD-aptamer conjugate, serving both as a fluorescence imager and a drug delivery carrier (Bagalkot et al. 2007). Dox was loaded into QD-aptamer conjugate *via* physical intercalation within the aptamer strand. Once internalized inside cancer cells, the QD-aptamer-Dox system gradually released Dox (for targeting therapy) and recovered the fluorescence of the QD (for synchronous cancer imaging).

3.6.2 Anti-HIV-1 gp120 RNA Aptamer

As described in sect. 3.1.4, the HIV-1 gp120 envelope protein represents an attractive molecular target for receptor-mediated drug delivery. Several 2′-F modified RNA aptamers have been isolated against HIV-1 gp120, which can specifically bind and be rapidly internalized into HIV-1 infected cells (Zhou et al. 2008, 2009). Using an afore-mentioned strategy described by Giangrande, a dual-action anti-gp120 aptamer-siRNA chimeric RNA was developed (Zhou et al. 2008; Neff et al. 2011).

This dual function chimera blocked HIV-1 infectivity and also selectively delivered siRNAs into HIV-1 infected cells which suppressed HIV-1 replication. Continued efforts to improve the aptamer delivery resulted in an aptamer-mediated combinatorial multi-targeting RNAi therapeutic, in which a single anti-gp120 aptamer was tightly tethered with three different siRNAs targeting HIV-1 *tat/rev* transcripts and HIV-1 host dependency factors (CD4 and TNPO3) through a "sticky" bridge (Zhou et al. 2009). The resulting aptamer-stick-cocktail siRNA conjugates suppressed viral loads in cell culture and *in vivo* in a humanized mouse model for HIV-1 infection (Zhou et al., unpublished).

3.6.3 Anti-PTK7 DNA Aptamer

Protein tyrosine kinase-7 (PTK7) is a member of the receptor tyrosine kinase family, which is highly expressed during thymocyte development in both mice and humans. Receptor protein tyrosine kinases transduce extracellular signals across the cell membrane. Tan and colleagues have isolated a panel of DNA aptamers against CCRF-CEM cells using a whole cell-SELEX (systematic evolution of ligands by exponential enrichment) procedure (Shangguan et al. 2006). One of the selected DNA aptamers, sgc8 identified to target PTK7, has been demonstrated to be specifically taken up into lymphoblastic leukemia T-cells *via* a receptor-mediated endocytosis (Shangguan et al. 2007). Recent studies have exploited the anti-PTK7 aptamer as a promising targeting ligand for targeted drug delivery. For example, an anti-PTK7 aptamer with a thiol group was covalently attached to Dox *via* an acid-labile linkage (Taghdisi et al. 2009). Once the aptamer-Dox conjugates specifically internalized into target cancer cells, Dox was easily released inside the acidic endosomal environment, selectively killing cancer cells. Recently, an anti-PTK7 aptamer-modified liposome was also used to facilitate targeted Dextran delivery (Kang et al. 2010). Additionally, an aptamer-conjugated Au-Ag nanorod (NR) was designed as a selective photo-thermal agent to efficiently kill tumor cells (Huang et al. 2008b).

3.6.4 Anti-NCL DNA Aptamer

Human nucleolin (NCL), a multifunctional protein involved in the synthesis and maturation of ribosomes, is over-expressed on the plasma membrane of several cancer cells such as breast, prostate, and lymphocytic leukemia. Moreover, NCL can transfer molecules between the cell surface and the nucleus. Therefore, it provides a potential target for the higher NCL-expressing cancer cell specific therapy. An anti-NCL DNA aptamer AS1411 (also known as AGRO100), a 26-mer guanine-rich oligonucleotide with high affinity and specificity to NCL, has been found to internalize into several cancer cell lines, including breast cancer cells (Bates et al. 1999; Soundararajan et al. 2008). A reversible AS1411 aptamer-liposome bioconjugate was developed to effectively deliver cisplatin to cancer cells and to improve therapeutic efficacy (Cao et al. 2009). Importantly, when a complementary antidote

was used to disrupt the aptamer's active structure, the cellular uptake of the aptamer-liposome was inhibited, thereby suggesting a novel controllable targeted drug delivery system. Most recently, Shieh et al. physically conjugated AS1411 with six molecules of a porphyrin derivative (TMPyP4), a broadly used photodynamic therapeutic agent (Shieh et al. 2010). By NCL-mediated internalization, the aptamer-TMP complex exhibited a higher TMPyP4 accumulation and specific photo-damage in MCF7 breast cancer cells.

3.7 Other Internalizing Molecule-Mediated Drug Delivery

In addition to the afore-mentioned more popular internalizing molecules, other specific ligands have been applied for targeted drug delivery.

Anisamide has served as a targeting ligand for tumor cells expressing the sigma receptor, a transmembrane protein that plays a role in regulating ion channels. It was found that sigma receptors are over-expressed in a diverse set of human and rodent tumor cell lines (Vilner et al. 1995). Small molecules such as asanisamide, haloperidol and opipramol have been reported as sigma receptor ligands and have been developed as radio-imaging agents for tumors (Maurice and Su 2009; Cobos et al. 2008; John et al. 1999). These receptor-specific ligands can be linked to various nanocarriers, or be directly conjugated to the oligonucleotide itself. For example, Huang and colleagues have successfully conjugated the high-affinity sigma receptor ligand asanisamide to lipopolyplex nanocarriers for specifically delivering doxorubicin or siRNAs to tumors in animals (Li and Huang 2006; Banerjee et al. 2004). Their subsequent studies confirmed that the cellular uptake was mediated *via* a sigma receptor dependent pathway. Similarly, haloperidol-modified lipoplexes were shown to mediate tenfold greater delivery of DNA to breast carcinoma cells compared with the control lipoplexes (Mukherjee et al. 2005). Most recently, anisamine also was directly conjugated to antisense oligonucleotides (ONs) (Nakagawa et al. 2010). The trivalent anisamide-ONs conjugate significantly enhanced receptor-specific cell uptake and biological activity.

Hyaluronan, also known as hyaluronic acid (HA) or sodium hyaluronate, is a natural anionic polysaccharide which can be efficiently taken up into cells by HA receptor mediated endocytosis (Stern et al. 2006; Prevo et al. 2001). Therefore, HA and its derivatives have been widely used as novel targeting ligands as well as target specific, long acting drug carriers of various therapeutic agents, including Dox, paclitaxel, proteins, peptides and nucleotide therapeutics (Oh et al. 2010). For example, the HA-modified long-circulating liposomes actively targeted tumors over-expressing the HA receptor (Peer and Margalit 2004a, b). The HA-poly-L-lysine (PLL) conjugate was shown to target sinusoidal epithelial cells in the liver (Jiang et al. 2009). The delivery of siRNA with HA modified PEI significantly improved gene silencing in HA receptor over-expressing cells. Most recently, HA derivative-quantum dot (HA-QD) conjugates facilitated targeted delivery and accumulated more efficiently in the cirrhotic liver than the normal liver after tail-vein injection (Kim et al. 2010).

4 Conclusion and Perspectives

Although Paul Ehrlich's dream – "magic bullets" – did not stand the test of time one century ago, the creative concept of "targeted therapy" that drugs are capable of going straight to their intended targets has inspired generations of scientists to continually pursue targeted based delivery approaches to selectively treat human diseases. In addition to passive targeting, active targeting that is based on the avid and specific interaction between the targeting ligands and the molecular targets has drawn much attention. Today, in view of the tremendous efforts in developing targeted therapy, an increasing number of specific internalizing molecules have been adapted for active targeting of tumors or infectious diseases. Moreover, a better understanding of the molecular events associated with human diseases and the emergence of new technologies (such as nanotechnology, RNAi, Cell-SELEX) have greatly accelerated the drug discovery process over the years.

Despite these advances bringing these targeted therapies to the clinic is currently a slow process. For example, one important concern for using cell surface receptors for active targeting is to engineer the targeting ligands with therapeutic agents or their carriers without disrupting the receptor-binding ability or reducing the therapeutic efficacies of the drugs. In the regard, a precisely engineered nanocarrier with multiple functions may be considered a promising therapeutic system. Ideally, an intelligent multifunctional nanocarrier with combinatorial targeting ability can efficiently encapsulate multiple drugs, prolong circulation time, accurately accumulate and release drugs in the targeted cells/tissues, eventually achieving maximal therapeutic efficacy and providing custom/tailored treatments. Meanwhile, such multifunctional nanocarriers can be used to visualize their location in the body for real-time monitoring of therapeutic responses.

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