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Targeted Drug Delivery to the Nucleus and its Potential Role in Cancer Chemotherapy

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Abstract
Macromolecules and supramolecular complexes are frequently required to enter and exit the nucleus during normal cell function, but access is restricted and exchange to and from the nucleus is tightly controlled. Drug delivery vehicles developed to date mainly deliver drugs to endo/lysosomal vesicles rather than the nuclei. Cancer remains one of the leading causes of death worldwide. Although enticing, the concept of a chemotherapeutic treatment directed towards a single target that kills tumour cells, without any harmful side effects or death of neighbouring cells is probably naive, due to the fact that tumour cells arise from normal cells and share many common biological features with them. Various means to damage/destroy tumour cells preferentially have been developed, but as yet, none are truly selective. However, by combining numerous tumour-specific/-enhanced targeting signals into a single modular multifaceted approach, it may prove possible sometime in the future to achieve the desired outcome, without any unwanted bystander effects, with the delivery of cytotoxic drugs/DNA directly to the nucleus specifically within tumour cells of great interest in this context. Herein, we discuss the biological barriers and summarize the recent progresses of nuclear drug delivery for chemotherapy, emphasizing strategies that appear useful for rational design of vehicles for in vivo tumor cell nuclear targeted drug delivery.

Key words: *Nuclear targeted drug delivery, biological barriers, Tumour cells, Chemotherapy.*

1. INTRODUCTION

In the case of drug delivery, the nucleus is also one of the most sensitive sites for drug-induced damage, such as in the case of photosensitising agents used in photodynamic therapy. Photodynamic therapy relies upon the specific activation of photosensitisors such as protoporphoryn IX or chlorine 6 using longwave-length light, releasing singlet oxygen species, the cytotoxic effects of which do not usually exceed 40 nm from the site of activation [1]. Since the nucleus is a hypersensitive site for active oxygen species-induced damage, coupling of the optimised T-ag NLS (nuclear localizing signals) to chlorine 6-containing complexes, either cross-linked to a carrier or encoded as part of a fusion protein can result in a 2000-fold reduction of the EC50, highlighting the importance of specific nuclear delivery of these chemical agents [2]. In terms of cancer therapy, delivery of these active agents specifically to the nucleus of tumour, but not normal cells, is the key to their being “magic bullets” in terms of effective and safe anti-tumour therapeutics.

Drug delivery vehicles developed to date mainly deliver drugs to endo/lysosomal vesicles rather than the nuclei. The drugs (or with their vehicles) have to escape from endo/lysosomal vesicles into the cytoplasm [3] and then translocate into the nuclei. However, cancer cells have many intracellular resistance mechanisms to limit the access of cytosolic drugs to the nucleus by such mechanisms as over expression of drug efflux pumps (e.g. P-gp), drug metabolism and detoxification, drug sequestering to acidic compartments, and drug deactivation [4-5]. As a result, only a small percentage of drugs delivered into the cytosol finally reach the nucleus in drug resistant cells. For example, only 5–10% of covalently bound cell-associated cisplatin is found in the DNA fraction, whereas 75–85% of the drug binds to proteins [6-7]. Therefore, efficient nuclear drug delivery is apparently an effective approach to increase their therapeutic efficacy. Herein, we discuss the nuclear transport mechanism and its regulation, importance of drug delivery to nucleus and summarize the recent progresses of nuclear drug delivery for chemotherapy, emphasizing strategies that appear useful for rational design of vehicles for in vivo tumor cell nuclear targeted drug delivery.

2. NUCLEAR DELIVERY OF DRUGS: LESSONS LEARNED FROM VIRUSES

One of the most effective proposed methods to effect efficient tumour cell killing is to target drugs specifically to

tumour cells as opposed to normal cells, and then subsequently to direct them to hypersensitive subcellular sites within these cells, such as the nucleus [8] . This should help to accomplish the ultimate goal of being able to avoid harmful side effects on the surrounding normal healthy cells, without compromising on cytotoxicity towards tumour cells. However, in order to achieve this, one needs to overcome a series of natural cellular barriers (see Fig. 1), which prevent the entry of foreign material into all cells and more importantly into the nucleus within these cells [9].

Viruses have evolved specific mechanisms to overcome such barriers, with many relying on the delivery of the viral genome into the nucleus of host cells as an essential part of their life cycle [10]. However, safety considerations such as pathogenicity, oncogenicity and the stimulation of an immune response have hampered their use in clinical settings. To overcome these issues, much progress has been made in the development of viral mimics or modular recombinant transporters (MRTs) that imitate viruses by replicating/retaining all of the necessary cellular and subcellular targeting functions of the virus as a whole, but without the associated safety concerns [11].

2.1. Cellular barriers to nuclear delivery

The first major barrier to nuclear drug delivery is the plasma membrane, which surrounds all mammalian cells and restricts the passage of large hydrophilic or charged molecules[12] . Thus, the first step in drug delivery is cell entry, which must involve binding to and passage through the cellular membrane. To facilitate this, many viruses recognize specific receptors expressed/exposed on the extracellular surface of target cells. Depending on the virus or vector in question, receptor binding may result in subsequent internalisation into an endosome, as is the case for non-enveloped DNA viruses such as adenovirus [13] .

Endosomal entrapment ultimately results in degradation of the enclosed ligand when the endosome fuses with a lysosome [14]. To avoid this, many viral proteins also function as endosomal escape moieties, undergoing conformational changes in response to the decreased pH found in endolysosomes, thus disrupting the vesicle membrane and facilitating release of the virus into the cytoplasm [8] , [13] . The virus must subsequently traverse the cytoplasm through the crowded network of the cytoskeleton, avoid degradation, and translocate into the nucleus in order to be expressed and replicated [9], or in some cases, integrated into the host cell genome, as is the case for retroviruses. Nuclear transport is known to be the most rate-limiting step in this process, implying that the nuclear envelope represents the most substantial barrier for gene or drug delivery to cells, including cancer cells [15].

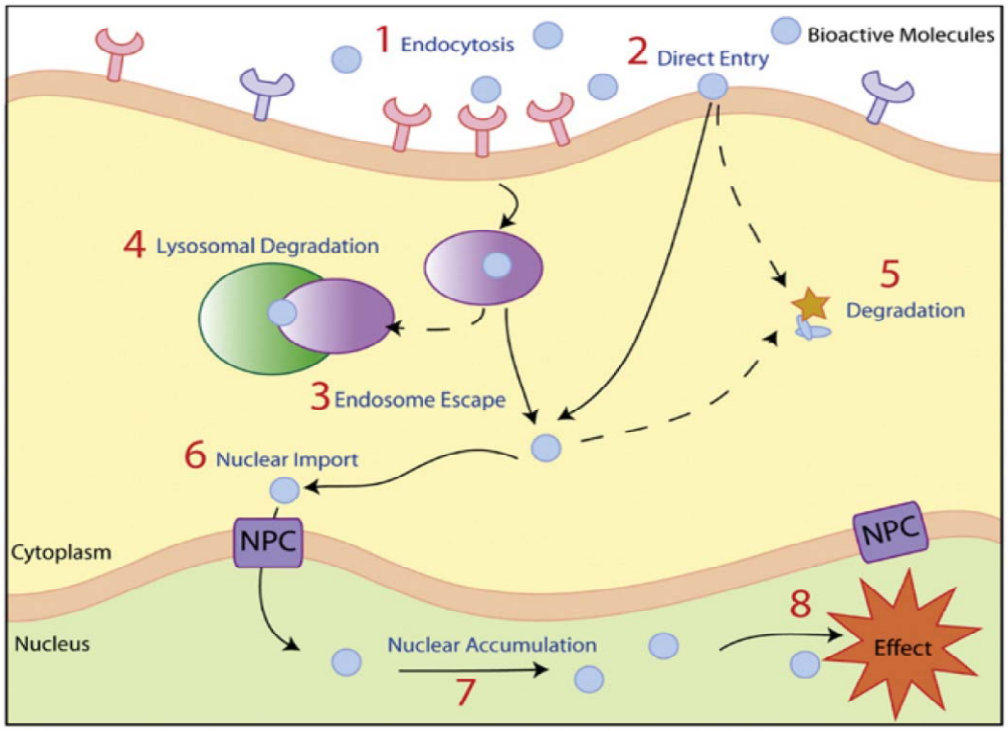

Fig. 1. Cellular barriers to bioactive molecules/nanocarriers. Schematic of the cellular barriers faced by bioactive molecules such as drugs or therapeutic DNA en route to the nucleus. The plasmamembrane represents the firstmajor barrier to bioactive compounds and can be overcome through the use of ligands to target cell-surface receptors, resulting in an endocytoticmethod of uptake (1) or through direct entrymechanisms such as the use of protein transduction domains or electroporation (2). Once inside the cell escape from endosomes is necessary (3), if taken up by endocytosis, in order to escape degradation (4), whilst the numerous degradative enzymes in the cytosol must also be avoided (5). To access the nucleus, transport through the nuclear pore complexes (NPC) embedded in the nuclear envelope (6) is necessary, to enable ultimate accumulation of the drug/DNAwithin the nucleus (7), to lead to a biological effect (8).

2.2. Nuclear transport mechanisms

The nucleus is the control centre of the eukaryotic cell, being both the site of storage and replication of the cell's genetic material, and of processes such as transcription and ribosome assembly that are central to synthesising the cellular complement of proteins that carry out all of its functions. The nucleus is separated from the rest of the cell cytoplasm by the double membrane structure of the nuclear envelope, which is punctuated by nuclear pore complexes, through which all transport between the nucleus and cytoplasm occurs [16]. The nuclear pore complex has a tripartite structure, consisting of a central channel and a cytoplasmic and nuclear ring, which are made up of more than 50 different proteins known as nucleoporins [17]. Each individual nuclear pore complex is the location of approximately 1000 translocation events every second, in either direction, consisting of both active and passive events [18]. Small molecules are able to pass through the nuclear pore complex via passive diffusion, but molecules N~45 kDa require specific targeting signals to gain either access to or egress from the nucleus [19].

In the import direction, nuclear localisation signals (NLSs) are recognised by members of the Importin (also known as karyopherin) superfamily of cellular nuclear transport proteins, of which there are two main types, α and β [17] . NLSs themselves fall into one of two categories, classical or non-classical sequences [20]. The best understood types of classical NLS are those that are typified by the NLS of the simian virus 40 large tumour antigen (T-ag: PKKKRKV132; single letter amino acid code, basic residues in bold), which is a monopartite NLS comprising a single stretch of basic amino acids [21], [22], or that of the Xenopus protein nucleoplasmin (KRPAATKKAGQAKKKK170), which is bipartite and consists of two short stretches of basic amino acids separated by a 10– 12 amino acid spacer[23]. Non-classical NLSs on the other hand, such as the largely hydrophobic 38-residue M9 sequence of the human mRNA-binding protein hnRNP [24] lack runs of basic amino acids.

Conventional nuclear transport (Fig. 2) involves recognition of an NLS by the Importin α subunit of the Importin α/β heterodimer [25], followed by docking at and translocation through the nuclear pore complex mediated by the Importin β subunit[26], [27]. Once inside the nucleus, the monomeric guanine nucleotide binding protein Ran, in its GTP bound form, binds to Importin β to actively displace Importin α and effect release of the cargo into the nucleoplasm [28] . Importin β itself and its numerous homologues are also able to mediate nuclear transport of many cargoes in an analogous fashion, but without the need for Importin α[9]. In humans, N20 homologues of Importin β have been described, whereas 6 Importin αs are known, which differ in both their cargo specificity as well as tissue distribution patterns[29]. Nuclear export (Fig. 2) is an analogous process to nuclear import, requiring nuclear export signals (NESs), which are recognised by Imp β homologues known as Exportins, of which Exportin-1 (CRM-1) is the best known example, largely through the fact that its activity can be specifically inhibited by the drug Leptomycin B [30].

2.3. Regulation of nuclear transport

Since numerous proteins need to be shuttled into/out of the nucleus in precisely regulated fashion to perform various roles in response to differing stimuli and throughout the cell life cycle, and given that many proteins contain more than one subcellular localisation signal, nuclear transport pathways can be regulated in numerous ways [19]. The best characterised of these regulatory means is through phosphorylation of cargo proteins, usually of sequences close to the NLS/ NES, resulting in either enhanced or diminished affinity for the requisite

Importin/Exportin protein[31] . The first reported example of regulated nuclear import of a protein was that of T-ag, whose subcellular distribution is regulated by several phosphorylation sites adjacent to the NLS, including a protein kinase 2 recognition site located 14 amino acids upstream (SSDDE115), which when phosphorylated at S111/112 significantly increases the affinity of the T-ag NLS for the Importin α/β heterodimer, resulting in enhanced nuclear import [32]. Similar regulatory mechanisms have also been identified in other Importin α/β-recognised NLS-containing proteins[33] Nuclear export of proteins can also be similarly regulated by phosphorylation, such as in the case of FOXO-1, where phosphorylation near to a CRM-1 recognised NES promotes nuclear export by stabilising the FOXO-1/ CRM-1 interaction, or Pho4, where phosphorylation at S114/128 facilitates recognition by Exportin-4, resulting in nuclear export [34]. NLS-Importin interactions can also be regulated by other mechanisms, including regulation of Importin expression levels and tissue distribution.

3. NUCLEAR DELIVERY OF DRUGS AND ITS ROLE IN CANCER CHEMOTHERAPY

One of the most effective proposed methods to effect efficient tumour cell killing is to target drugs specifically to tumour cells as opposed to normal cells, and then subsequently to direct them to hypersensitive subcellular sites within these cells, such as the nucleus[8]. This should help to accomplish the ultimate goal of being able to avoid harmful side effects on the surrounding normal healthy cells, without compromising on cytotoxicity towards tumour cells. However, in order to achieve this, one needs to overcome a series of natural cellular barriers (see Fig. 1), which prevent the entry of foreign material into all cells and more importantly into the nucleus within these cells[9].

Challenges of nuclear delivery for cancer chemotherapy

The entire process of in vivo cancer cell-targeted nuclear drug delivery can be broken down into a series of steps, each of which has considerable resistance or biological barriers critical to the successful delivery, as shown in Fig. 2. This process is very similar to gene delivery. How to overcome these barriers to achieve efficient gene delivery has been extensively reviewed elsewhere[35].

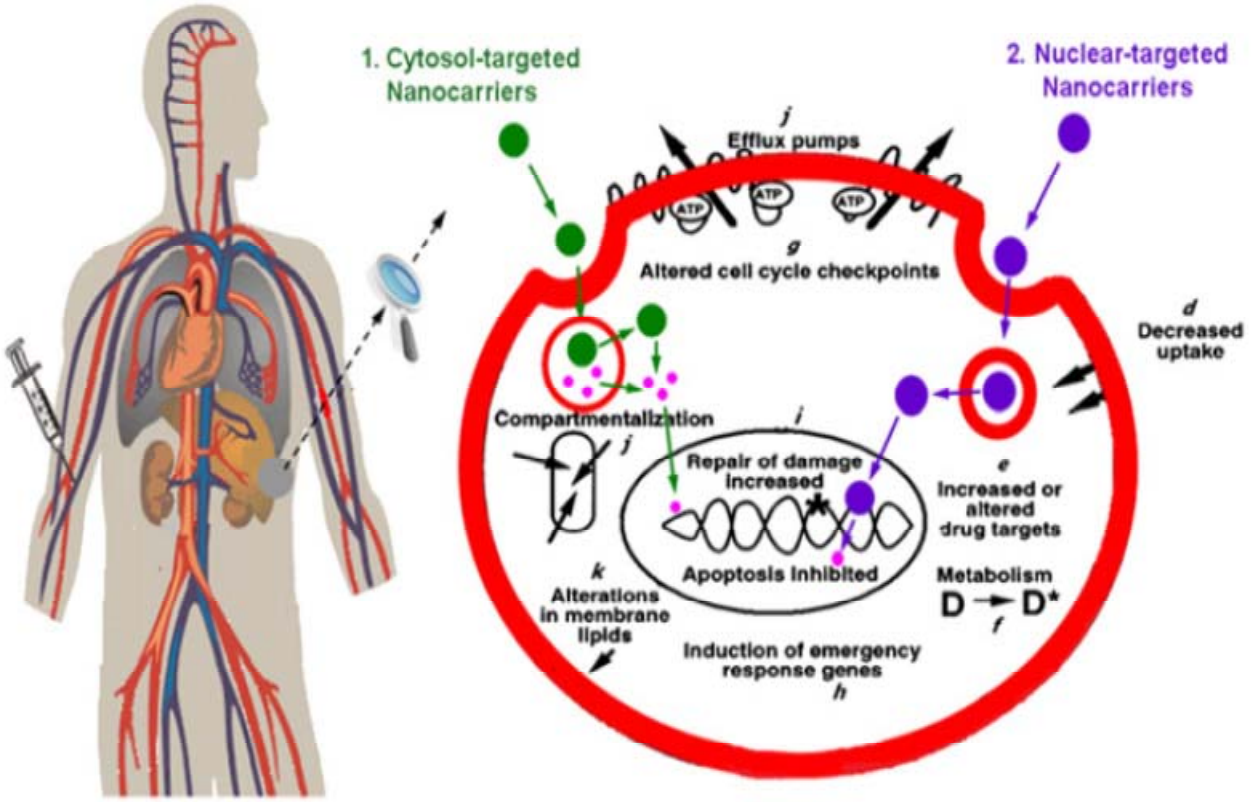

Fig. 2. Cancer nuclear drug delivery. After i.v. administration, the nanocarrier circulates in the bloodstream and may accumulate in the tumor tissue via the EPR effect. The nanocarrier is generally endocytosed into an endo/lysosome. Subsequently there are two pathways for nuclear drug delivery: (1) Indirect nuclear drug delivery — cytosoltargeted drug delivery followed by nuclear accumulation; that is, either the carried drug is released from the nanocarrier into the endo/lysosome and then into the cytosol, or the nanocarrier escapes from the endo/lysosome into the cytosol and slowly releases the drug. Some of the cytosolic drug molecules then translocate into the nucleus;\ (2) Nuclear-targeted drug delivery: the endocytosed nanocarrier escapes into the cytosol, travels through the cytoplasm, localizes in the nucleus, and then releases the carried drug. Some drug resistance mechanisms (d–k) are also shown.

Similarly, a carrier for effective in vivo nuclear drug delivery should be carefully designed and equipped with appropriate "strategies" to overcome all of these potential obstacles[36]. For instance, cellular level (in vitro) nuclear drug delivery is well-documented. Drugs or nanocarriers functionalized with nuclear localizing signals (NLSs) or even simple cationic polymers can efficiently enter cells and localize in their nuclei, as discussed afterward. However, while these systems may be occasionally used for localized cancer treatments [37], they cannot be used for intravenous administration because they cannot overcome the extracellular barriers. Once in the bloodstream, cationic charges can cause strong non-specific cellular uptake and severe serum inhibition, and be rapidly cleared from the plasma compartments [38].

4. STRATEGIES FOR NUCLEAR DELIVERY OF CHEMOTHERAPY

Many systems in literature claimed "nuclear drug delivery" by showing enhanced nuclear accumulation of drugs to cancer cells in in vitro or in vivo models. According to the process that the intracellular drug localizes in the nucleus, we can classify all the reported "nuclear drug delivery" systems into two types (see Fig. 2). Some carriers enter into endo/lysosomes. They either release the carried drugs or escape into the cytosol and then release the drugs. The drug molecules released into the cytoplasm then have to, by their own, translocate from the cytosol to the nucleus (Fig. 2(1)). This cytosol-targeted drug delivery followed by drug nuclear localization is indirect nuclear drug delivery, and is achieved by increasing cytosolic drug concentration to facilitate the drug nuclear accumulation. On the contrary, in some systems the nanocarriers carry the drugs all the way to cancer cells, then cross through the cell membrane and the cytosol, and finally localize in the nucleus, where the drugs are then released (Fig. 2(2)).

We thereby name it nuclear-targeted drug delivery. Please note that the extracellular processes of the two types of nuclear drug delivery are the same. Below we first summarize the indirect nuclear drug delivery and then the developments of the nuclear-targeted delivery with emphasis of its rational design of nanocarriers for in vivo applications.

4.1. INDIRECT NUCLEAR DRUG DELIVERY

4.1.1. Cytosol-targeted drug delivery followed by nuclear drug accumulation

Intracellular free drugs can diffuse or re-distribute into the nucleus driven by the concentration gradient. Some drugs even have an inherent tendency to translocate to the nucleus. A mostly recognized example, such as doxorubicin (DOX), effectively accumulates in the nucleus by formation of a DOX–proteasome complex [39]. Shi et al. even demonstrated that DOX conjugated on a nanocarrier could lead it to the nucleus. Therefore, approaches that increase drug influx and/or decrease intracellular drug efflux will build up a high cytosolic drug concentration and therefore facilitate nuclear drug accumulation (Fig. 2 (1)). Indeed, many cytosol-targeted drug delivery systems capable of fast cellular uptake (drug influx) or inhibiting membrane-associated drug resistance (drug efflux) showed enhanced drug accumulation in nucleus, as summarized in the following session. We here only include the reports that claimed "nuclear drug delivery" and also showed direct evidence of enhanced nuclear drug accumulation. Furthermore, since the drugs distribute in both cytosol and nucleus, the eventual drug efficacy may be related to both cytosolic and nuclear actions of the drug.

4.1.2. Overcoming membrane-associated drug resistance

As discussed in Section 1, resistant tumor cells overexpress P-gp pumps in their membrane actively transporting drugs out of the cell while they diffuse through the cell membrane (Fig. 2j), leading to low cytosolic and thereby lownuclear drug concentrations. Nanocarriers are generally taken up into the cells by endocytosis, and thus drugs in the nanocarriers can bypass the P-gp pumps and be "smuggled" into the cells. Co-delivery of multidrug resistance (MDR) inhibitors or MDR siRNA to inactivate or silence the MDR further blocks the efflux of cytosolic drug by P-gp pumps. These approaches, separately or simultaneously, may greatly increase the cytosolic drug concentration and subsequent nuclear drug accumulation.

Cuvier et al. showed thatwhile free DOX could not enter MDR tumor cells, DOX-loaded nanospheres (DOX-NS) circumvented multidrug resistance and delivered a high concentration of DOX to the cell cytosol and nucleus. They proposed that DOX-NS were not recognized as a drug by P-gp, perhaps due to their molecular structure or the ionic charge [40]. Addition of active targeting molecules could further increase intracellular drug delivery efficiency. For instance, Elbayoumi and co-workers modified DOX-loaded long-circulating liposomes (Doxil) with the nucleosome-specific monoclonal antibody 2C5 (mAb 2C5) recognizing the tumor cell surface-bound nucleosomes . These mAb 2 C5-modified DOX-loaded PEGylated liposomes significantly enhanced nuclear drug accumulation and increased toxicity compared to the control to DOX-resistant colon cancer cell line, evidently bypassing the P-gp-mediated resistance [41] . Moreover, folic acidtargeted liposomes (FTL DOX) delivered a higher drug concentration to the whole MDR lung cancer cells and also their nuclei than free DOX. Importantly, in a subsequent in vivo adoptive assay, FTL DOX showed significantly better tumor inhibition than free DOX [42]. The mechanismfor this is unclear but itwas likely that part of the liposomal drug was in a different physical form from free drug. Aggregation or molecular stacking due to self-association of the intracellular liposomal DOX was a possibility, because DOX dimerization occurs at concentrations greater than 10mM [43-45]. This would explain the inability of cells to pump out liposomal DOX [42]. Sustained inhibition of P-gp activity to block the drug efflux may also efficiently increase intracellular as well as subsequent nuclear drug accumulation. Kabanov et al. showed that amphiphilic triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), PEO-b-PPO-b-PEO (Pluronic) very effectively sensitized MDR tumors to antineoplastic agents [46], as a result of two processes acting in concert: i) intracellular ATP depletion, and ii) inhibition of ATPase activity of drug efflux proteins. For instance, one of the copolymers, P85, was shown to cause an inhibition of multidrug resistanceassociated proteins (MRP) ATPase activity and induce ATP depletion. Considerable increases of vinblastine and doxorubicin accumulation in the cells overexpressing MRP in the presence of P85 and enhanced cytotoxicity of the drugs were thus observed [47]. These results were confirmed by in vivo study on animal models [48] and also clinical trials [49].

Nanoparticles-mediated simultaneous co-delivery of MDR inhibitors including tariquidar [50], curcumin [51], GG918 [52] and vitamin E TPGS [53] with drugs is also used to overcome the drug resistance for high cytosolic drug concentration. For example, the DOX and GG918 co-encapsulated polymer-lipid hybrid nanoparticles (PLN) demonstrated the greatest DOX uptake and anticancer activity to the MDR cells, while co-administration of two single-agent loaded PLN was least effective. Thus, the simultaneous delivery of DOX and GG918 to the same cellular location was critical in determining the therapeutic effectiveness of this anticancer drug-chemosensitizer combination [52].

Directly knockdown of MDR by siRNA was recently developed to enhance intracellular drug accumulation in resistant tumors [54–57]. Biocompatible, MDR1 siRNA loaded lipid-modified dextran-based polymeric nanoparticles efficiently suppressed P-gp expression in the drug resistant osteosarcoma cell lines, with increased drug accumulation in MDR cell lines [58]. Similarly, siRNA-mediated MDR-1 gene silencing at 100 nM dose in multidrug resistant SKOV3TR human ovarian adenocarcinoma cells significantly enhanced the cytotoxic activity of nanoparticle-encapsulated PTX to SKOV3TR cells probably due to an increase in intracellular drug accumulation upon MDR-1 gene silencing [59]. In another study conducted by Pakunlu et al., encapsulation of DOX into liposomes significantly increased nuclear localization of DOX and the drug-induced cytotoxicity to human MDR ovarian carcinoma cell line A2780/AD. Interestingly, co-delivery of DOX and antisense oligonucleotides simultaneous targeted to MDR1 and BCL2 mRNA with PEGylated liposome further significantly increased the nuclear accumulation of DOX [60].

4.1.3. Increasing drug cellular uptake by active targeting

As mentioned earlier, stealthy surface such as PEGylated nanocarriers is used to protect nanocarriers from the RES for long blood circulation time. However, it also substantially reduces the carriers' cellular uptake rate. Grafting nanocarriers with biorecognition molecules (ligands) whose receptors are overexpressed on tumor cell surface leads to receptor-mediated endocytosis [61] and thus promote their cellular uptake. Such ligands including folic acid, peptides, antibodies, transferrin and some other moieties [62] have been shown to contribute to nuclear delivery of various chemotherapeutic drugs.

For instance, many malignant tumor cells overexpress folate receptor (FR), whereas the access to the FR expressing in normal tissues is severely limited due to its location on the apical (externally facing) membrane of polarized epithelia [63]. In our recent studies, we found that the nanoparticles with FA targeting groups (TCRNs) were internalized much faster into SKOV-3 cells (FR overexpressing cell line) and subsequently, more efficiently localized into the nucleus than the nanoparticles without FA moieties. Particularly, TCRNs loaded with DOX (TCRNs/DOX) were more effective in killing SKOV-3 cells than free DOX [64]. Further, we also demonstrated that attachment of FAtargeting moieties significantly enhanced the cellular uptake and nuclear localization of CPT conjugated to latently amidized poly(Llysine) and PAMAMin FA-overexpressing tumor cell lines [65,42]. This FA-mediated enhancement of nuclear drug delivery was also observed in other drug-loaded nanosystems including DOX or Pt (IV) carrying liposomes [66], single-wall carbon nanotube [67], and D-α-tocopheryl PEG succinate (TPGS) [38].

Shi et al. recently functionalized an amphiphilic copolymer, poly (TMCC-co-LA)-g-PEG, with both anti-HER2 antibodies and DOX [68]. Active targeting through this antibody to HER2-overexpressing SKBR-3 cells and nuclear localization led by DOX were clearly demonstrated. The DOX and anti-HER2 dual functionalized nanoparticle exhibited tumor specific-targeting ability and was more efficacious against tumor cells than the nanoparticle formulation with either DOX or anti-HER2 alone. Khandare et al. demonstrated effective nuclear entry and enhanced antitumor activity of CPT by targeting luteinizing hormonereleasing hormone (LHRH) receptors in vitro and in vivo [69]. Hepatocytes express a large amount of asialoglycoprotein receptors on their surface, which could recognize galactose-terminated glycoproteins and internalize them into the cell interior [70,71]. Inspired by this mechanism, nanocarriers using galactose to target liver have been developed for cancer therapy. For instance, Wei et al. recently prepared 10-hydroxycamptothecin (HCPT) nanocrystallites and found that galactosylated chitosan located on the HCPT nanocrystallites enhanced the liver-targeted cellular uptake through an asialoglycoprotein receptormediated pathway. Thesenanocrystallites also exhibited the advantages of nuclear entry and active HCPT delivery, and consequently better anticancer cytotoxicity was achieved [72]. Active targeting of nanocarriers to prostate specific membrane antigen (PSMA) [73], integrin (i.e. RGD4C) [74], transferrin [75], fibroblast growth factor receptor (FGFR) and epidermal growth factor receptor (EGFR) were also shown to promote nuclear delivery of chemotherapeutic drugs.

4.1.4. Enhanced endo/lysosomal drug release

In contrast to low molecular weight drugs, which are internalized inside the cells by passive diffusion, nanocarriers are internalized by endocytosis. As the internalized carriers progress through the endocytic pathway, they encounter compartments, namely early endosomes, late endosomes and lysosomes, of progressively increasing acidity route toward lysosomes. The pH of early endosomes is typically near 6, the pH of late endosomes is near 5 and that of lysosomes is about 4 to 5. The acid pH and related enzymes in these compartments are extensively used to trigger drug release from the carriers for the drugs to “diffuse” into the cytosol and nucleus (Fig. 2).

Hydrazone, which is very stable at the physiological pH but quickly hydrolyzes at the lysosomal pH, is a mostly used acid-labile linker for conjugation of drugs to polymer carriers. For example,DOX was conjugated to PAMAM dendrimers via pH-sensitive hydrazone (named as PAMAM-hyd-DOX) or pH-insensitive linkers (named as PAMAMamide- DOX). The distribution of PAMAM-amide-DOX was found to be mainly cytosolic. However, the PAMAM-hyd-DOX conjugates efficiently released DOX, resulted in more nuclear accumulation of DOX and more cell death. Xiong et al. conjugatedDOX to the degradable PEOb-PCL core using the pH-sensitive hydrazone bond, namely RGD4CPEO- b-P(CL-Hyd-DOX), or using the more stable amide bonds, namely RGD4C-PEO-b-P(CL-Ami-DOX). The extent of intact DOX release from the first systemwas significantly higher than of the second one at pH 5.0. The first system demonstrated capability of DOX delivery to the nucleus similar to freeDOX in tumor cells,while the second formulation led to accumulation of DOX in the cytoplasm. In addition to the acid labile bonds, lysosomal degradable peptides (e.g. GFLG), which are cleavable by lysosomal enzymes to release the drugs, are also used for drug conjugation.

Lysosomal pH has also been used to trigger the drug release from pH-sensitive nanoparticles. Bae et al. used pH-responsive polyhistidine- block-PEG (PHis-PEG) to make micelles soluble at lysosomal pH (PHSM/f). DOX delivered by PHSM/f was found uniformly distributed in the cytosol as well as in the nucleus, while the non-pH sensitive micelles were entrapped in endosome and multivesicular bodies. The pH-sensitive PH is-based nanoparticles dissolved and released the drug into the lysosome. The used PHis, which is known to have an endosomal membrane-disruption activity induced by a “proton sponge” mechanism, disrupted the compartment membrane and released DOX into the cytosol. As a result, DOX/PHSM/f showed much higher in vitro and in vivo anticancer activities towards DOX resistant cells. In addition, polyacids-containing polymers may form pH-sensitive nanoparticles that display a lysosomal pH-responsive drug release. For instance, amphiphilic poly(N-

isopropylacrylamide co-N,N-dimethylacrylamide-co-10-undecenoic acid) self-assembled into pH- and thermal-responsive nanoparticles, which rapidly released DOX at the lysosomal pH and fast nuclear drug accumulation, compared to that by non-pH sensitive nanoparticles .

On the other hand, the harsh environment of lysosomes can easily degrade drugs sensitive to acid or these enzymes. Therefore, methods to rupture the lysosomal membrane have been proposed to release the drugs or the carriers into cytosol. Polycations, such as lipopolyamines, polyethylenimine and polyamidoamine can buffer the acidic endo/lysosomal pH and thus cause rupture of endo/ lysosomal membrane. This process, referred to as proton sponge effect proposed in gene delivery, may be used in drug delivery. For instance, the presence of cationic polyamines (PAH) in FMMSNs (fluorescent monodisperse mesoporous silica nanospheres) was proposed to enhance endosomal escape of FMMSN, as further verified by acid base titrations experiments.

Some specially designed polyacids, such as poly(propylacrylic acid) (PPAA), are shown to disrupt endosomes at pH 6.5 or below, causing the cytosolic release of cargo molecules. Other endosome-disrupting agents, such as HA2, chloroquine and a large family of anionic polymers, may be attached to the surface of drug loaded carriers for endosomal destabilization, cytoplasmic escape and subsequent nuclear entry [76].

4.1.5. Intracellular drug compartmentalization and nuclear accumulation

Mammalian cells are extensively compartmentalized and membrane bound compartments/organelles occupy over half of the total cell volume. Therefore, once the drug molecules are released into cytosol, they will quickly distribute among the cytosol and compartments/organelles governed by the properties of the drug and the intracellular environment.

The nuclear envelope has numerous pores that allow for free diffusion of small, low-molecular weight molecules to and from the cytosol. However, a drug must be nucleotropic in order for the drug molecules in cytosol to predominantly accumulate to the nucleus.

The binding of drugs to nuclear DNA or other biomolecules is the major approach for most anticancer drugs to achieve neucleotropic ability. The best examples are DNA binding drugs, such as the anthracyclines (e.g. doxorubicin) and cisplatin. Moreover, drug nuclear accumulation is strongly affected by characteristics of tumor cells. For instance, drug resistant cells can develop many mechanisms to alter the drug compartmentalization in cytosol and nuclear accumulation. One such better-understood mechanism is the pH partitioning-mediated selective accumulation of weakly basic drugs, such as anthracyclines and camptothecin and its derivatives, within acidic organelles particularly lysosomes and Golgi. Weakly basic drug molecules with appropriate pKa values mostly exist in their un-ionized, membrane-permeable form in the neutral cytosol. Upon crossing the lipid bilayer and entering the acidic lysosomes, the drug molecules become protonated and thus membrane-impermeable, and cannot readily diffuse out of the lysosomes. This process allows lysosomes to sequester the drug molecules away from the cytosol and other compartments/organelles. The degree of drug sequestration into lysosomes (i.e. lysosome-to-cytosol concentration ratio) is predominantly determined by the pKa of the drug and the lysosome-to-cytosol pH differential. In some instances, lysosomal sequestration can account for nearly 100% of the total drug accumulation within a cell. Furthermore, drug resistant cells may also express transporters that alter the drug's nuclear accumulation. For instance, copper transporting P-type ATPase, ATP7A, preferentially ships DOX to the Golgi apparatus and enhances the efflux rates of DOX and SN-38. Removal of DOX from the nucleus is also presumably mediated by nuclear P-gp or other proteins involved in the nuclear-cytoplasmic trafficking and compartmentalization of drugs. Consequently, the indirect nuclear drug delivery relying on enhanced cytosol-targeted drug delivery may not be efficient for drug resistant cells.

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