Expert Opinion

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Subcellular targeting: a new frontier for drug-loaded pharmaceutical nanocarriers and the concept of the magic bullet

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The ability of a pharmacologically active molecule selectively to find its target is closely linked with its potential as a successful therapeutic drug. It has become increasingly evident that there are several pharmacologically active molecules that exert their action on molecular targets inside cell organelles. In the case of a drug molecule with no defined specificity for a particular organelle, the molecule would either need to have sufficiently long metabolic stability to allow for random interaction with the organelle to occur, or a targeting strategy for the intended subcellular compartment would need to be devised in order to potentiate therapeutic effect. In the case of molecules with a stronger affinity for a non-target subcellular compartment, there exists even greater need for the ability to control subcellular disposition. Subcellular or organelle-specific targeting has thus emerged as a new frontier in drug delivery. In this review selected examples of recent work are discussed that the authors believe might eventually lead to the application of pharmaceutical nanocarriers to create the next generation of 'magic bullets' that are capable of delivering a drug payload to a molecular target at a subcellular location.

Keywords: drug delivery, nanocarriers, subcellular targeting

Expert Opin. Drug Deliv. (2009) 6(11):1135-1148

1. Introduction

1.1 Re-examining the concept of a drug molecule as a magic bullet

Modern drug therapy is based largely on the paradigm that an ideal drug selectively exerts a desired pharmacological activity free from negative side effects to modulate either symptoms or the underlying biochemical cause of a disease, thus providing a benefit to the patient. To have such selective action, the drug molecule should ideally interact with only the disease-associated biochemical pathway, having no activity with respect to any normal biochemical pathway. This principle was famously explored by Paul Ehrlich in his search for agents with selective toxicity towards bacteria. Ehrlich's work is widely accepted to have given rise to the concept of the ideal drug molecule as a 'magic bullet', a term that he used for the first time in his Harben Lectures [1]. Finding such selective molecules is relatively easy when there are significant differences between the disease-causing process and normal human biochemical pathways, as in the case of infectious diseases. Not surprisingly, in the decades since Ehrlich's work infectious diseases have become much easier to treat, but even then the so-called lack of activity in non-disease cells is dose-dependent and not absolute. Most drugs that are considered to be selectively toxic to invading pathogens are in fact toxic to human cells as well, but just at higher doses. However, given that the new challenges in drug therapy lie in the treatment of diseases associated with malfunctions of normal human biochemical pathways in certain tissues, the concept of the magic bullet perhaps needs to be

10.1517/17425240903236101 © 2009 Informa UK Ltd ISSN 1742-5247

redefined or at least clarified. In the infectious disease example it is essential to understand that the so-called magic bullet did not necessarily have to home in on the disease agent, but could in fact accumulate to the same level in both host and pathogen cells. Just as long as the agent was toxic only to the pathogen it was considered by many to be a magic bullet. In fact, Ehrlich's Nobel Prize Lecture of 11 December 1908 speaks of 'outlining the principles of selective toxicity' rather than selective accumulation. Therefore, one could argue that for a molecule to be a magic bullet, it does not have to accumulate selectively at its intended site of action but just that it should not exert its action anywhere but at that intended site of action. This is different from what is now referred to as drug targeting.

The term 'targeting' is most often meant to imply that the molecule is in some way able to accumulate selectively at an intended site of action and that the selective accumulation is associated with its selective action as a magic bullet. Unfortunately (perhaps because of the widespread use of the noun target to describe a potential molecular site of action), there is often the misconception that a drug that is believed to act at a molecular target (noun) is by default also able to target (verb) or 'home in' on that target (noun). It would therefore be more appropriate to define a true magic bullet as a drug molecule that is specific in its activity for a molecular target but that is also able to accumulate selectively at this molecular target and exert a selective therapeutic action by virtue of both its specific activity and its selective accumulation. This distinction is important when considering the daunting challenge of developing magic bullets for diseases such as cancer, neurodegenerative diseases such as Alzheimer's, as well as hormone imbalance diseases such as diabetes, which are becoming more widespread. Unless unique molecular targets found exclusively (or at sufficiently higher levels) in the diseased state and not in normal state are discovered, magic bullets by the traditional definition or the compromise of dose-dependent activity at the site of action may not be feasible.

At a very basic level, selective accumulation is influenced by bioavailability and subsequent biodistribution. In the context of drug molecules, biodistribution is related primarily to physicochemical properties. Many potent drug candidates have low bioavailability owing to their limited water solubility. On the other hand, water-soluble compounds display a very limited ability to cross biological membranes, which essentially can exclude them from the cell interior. To overcome the limitations that a compound's physicochemical nature can impose on its potential pharmaceutical application, the process of large-scale screening of chemical libraries has been extended beyond just identifying desired bioactivity. Screening approaches routinely incorporate selection for desirable physicochemical properties that might confer high bioavailability as well. On the down side, this approach leads to many potent molecules being excluded from further development because they are not true magic bullets, that is, although they may have a potent pharmacological action at a desired molecular target, they are not able to find their way exclusively to that target. There is most certainly a growing list of such molecules that are in essence potential drugs if only a delivery strategy can be devised to get them to their molecular target in the human body. As the search for the perfect magic bullet continues, there is a significant effort to improve the action of available molecules by using targeted delivery approaches. It is not surprising that that the field of drug targeting has grown significantly in the effort to develop better therapy. Based on the experience over the decades since Ehrlich first introduced the magic bullet concept, it seems more reasonable to separate the functions of pharmacological action and selective accumulation into properties desired in a drug molecule and in a delivery system, respectively, rather than the traditional expectation that the drug molecule alone possesses both properties.

1.2 Pharmaceutical nanocarriers in the search for magic bullets

Generally speaking, drug disposition may be modulated by means of three broad approaches (**Figure 1**). First, the drug molecule might be subtly modified to change its physicochemical properties without adversely affecting its inherent pharmacological action. This is essentially the intent of medicinal chemistry approaches and the concept of structure activity relationship (SAR) studies that have now become standard practice and often are not even considered a means of achieving targeting. The second approach might be considered to be an extension of the first but is different in that it involves using chemistry to conjugate ligands that are often larger than simple organic functional groups to change the biodistribution of a molecule. Again this approach works as long as the conjugation does not adversely affect the desired pharmacological activity of the molecule. Conjugation using selectively cleavable linkers is an extension of this strategy. The third strategy involves the use of a carrier system and does not involve chemical modifications to the pharmacologically active molecule. Pharmaceutical nanocarriers fall into this category and several such technologies are being developed that are fast becoming applicable to a variety of pharmacologically active molecules.

Liposomes are arguably the prototype of all pharmaceutical nanocarriers now under development. These artificial vesicles prepared from phospholipids are able to trap hydrophilic drugs in the aqueous inner space and lipophilic drugs in the phospholipid bilayer. Liposomes were serendipitously discovered in the early 1960s [2-4] and proposed in the early 1970s as potential drug carrier systems [5-7]. About two decades later the US FDA approved Doxil® (Johnson & Johnson Corporation, NJ, USA) and Daunoxome® (Gilead Sciences, Inc., CA, USA) as the first liposome-based drug formulations. Doxil is a formulation of doxorubicin hydrochloride, whereas Daunoxome is a formulation of daunorubicin. In both formulations, the liposome surface is modified with polyethylene glycol to increase circulation time significantly following systemic administration. Long circulation time, in turn, is prerequisite for an increased

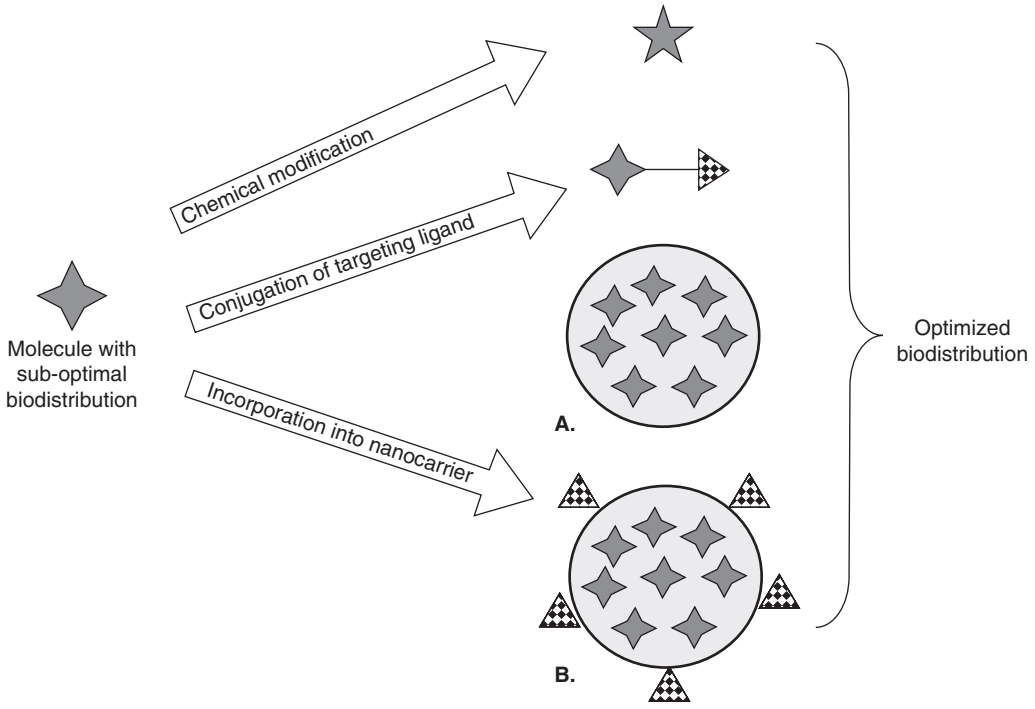

Figure 1. Major approaches to optimizing the disposition of a drug molecule. A. Non-surface-modified nanocarrier. **B.** Drug-loaded nanocarrier surface modified with targeting ligand.

accumulation of the liposomal drug at sites of vascular damage, such as in solid tumors and at inflamed areas. On entering the interstitium of a solid tumor by a mechanism known as the enhanced permeability and retention (EPR) effect [8], liposomes eventually disintegrate, resulting in the release of the drug from its carrier. Using such approaches it is now routinely possible to achieve tissue-specific as well as, in some cases, cell-specific preferential accumulation of a higher fraction of the administered dose. In many cases (as with Doxil and Daunosome), reducing the fraction of the dose that accumulates in non-target sites has a beneficial effect by reducing unwanted side effects. The altered biodistribution and bioavailability of the liposomal encapsulated drug also requires treating the liposomal drug as a new drug entity different from the free drug. This principle, already recognized > 20 years ago by the pioneers of Liposome Technology [9], most certainly applies to all nanocarrier-associated drugs now under development, giving the term 'magic bullet' a whole new dimension.

Pharmaceutical nanocarrier systems thus offer what might be viewed as a non-chemical approach to modifying the disposition of drug molecules. All chemistry can be performed on the components of the nanocarrier system that can then be loaded with the drug to afford targeted delivery. However, despite such advances, the improvement in drug action is not always dramatic. Many drugs act at molecular targets inside the cells and these molecular targets are often in well-organized subcellular structures inside the mammalian cell. There is arguably fractal symmetry between the case of drug delivery to a cell and drug delivery to a molecular target inside a subcellular compartment. The cell could be viewed as being a small, slightly simpler but nonetheless highly organized 'body' with 'organs' (organelles) and 'cells' (defined structures and molecular arrangements) within these organs. It should therefore stand to reason that controlling drug disposition within the cell might also be necessary for optimal drug action. Unfortunately, many drug delivery approaches seem to ignore this level of targeting. There often seems to be an assumption that mediating cell cytosolic internalization is adequate to ensure the interaction of the drug molecule with its final subcellular target by virtue of simple diffusion of the drug molecule and random interaction with various subcellular structures in the cell. However, it has become increasingly evident during the last decade that such an assumption cannot always be made [10-19]. Subsequently, the focus shifts towards subcellular drug targeting, that is, directing therapeutic agents to an individual organelle has become a new frontier [20]. Clearly the key to subcellular targeting lies in a thorough understanding of the mechanisms of subcellular transport and trafficking in order to identify potential targeting ligands and to develop targeting strategies. Although there is still much to be elucidated in this area of research, recent literature does offer some approaches that have already been explored for drug therapy. What follows is a description of some of these studies and a discussion based on the authors' experiences in the field of subcellular targeting to provide a preliminary

roadmap for developing the next generation of magic bullets based on nanocarriers.

2. A hitchhikers' guide to the design of subcellularly targeted pharmaceutical nanocarriers

2.1 Understanding the intracellular fate of low-molecular-mass drug molecules

The interior of a cell is very different from an aqueous buffer solution, in which small drug molecules can diffuse freely and interact randomly with potential co-solutes. In addition to the presence of the cytoskeletal network and various dispersed organelles, the cytoplasm contains a large amount of dissolved macromolecules. The concentration of dissolved macromolecules in the nucleoplasm and cytoplasm of living cells has been determined to be between 50 and 400 g/l [21,22]. Subsequently, transport or diffusion events in such a crowded solution cannot be expected to be the same as those in buffer solutions. Generally, intracellular diffusion has been characterized as hindered diffusion reflecting, among other factors, the high level of molecular crowding [23,24]. Also, the fluid-phase viscosity of the cytoplasm and binding to intracellular components are believed to influence the diffusion of solutes inside a cell [25,26].

Efforts aimed at thoroughly understanding cellular material properties such as cytoplasmic viscosity are underway [27]. Despite such constraints, the cytoplasmic diffusion of a low-molecular-mass compound is still measurable and can be expressed as the translational diffusion coefficient; however, as molecular size increases, diffusion is less likely to be the primary mode of transport. For example, using spot photobleaching it was possible to measure the movement of DNA fragments of different sizes following microinjection into the cytoplasm of HeLa cells [25]. Not surprisingly, the rate of diffusion decreased with increasing size of the DNA. In addition to molecular size-dependent diffusion, the subcellular fate of a drug is influenced by the extent to which the molecule might interact or even bind to subcellular components, such as membranes and cell organelles. These interactions obviously depend on the physicochemical properties of the drug. The ability to predict the influence of various properties of the drug molecule on the likely site of accumulation within the cell could prove to be a powerful tool in drug design.

Based on the intracellular distribution of a large variety of fluorophores, a quantitative structure activity relationship (QSAR) model for predicting cellular uptake and intracellular distribution of low-molecular-mass compounds has been proposed [28]. This QSAR approach was applied recently to identify potential common chemical features of molecules that are known to accumulate selectively at or inside mammalian mitochondria within living cells [29]. The QSAR approach has also proved useful for the modeling of cationic transfection lipids [30] and could therefore be applicable to predicting the subcellular disposition of a potential therapeutic molecule and even to designing molecules with a desired subcellular affinity for the development of subcellular targeting approaches.

2.2 Adapting nanocarriers developed for targeting at the cellular level to the subcellular level

Most pharmaceutical nanocarriers can be modified for some level of targeting to specific tissues if not specific cell types. Long circulating liposomes and nanoparticles are able passively to target areas of leaky vasculature by virtue of the EPR effect and can also be modified with antibodies or other targeting ligands to afford cell-specific recognition. The next logical step in the development of targeted nanocarriers would be to extend control over nanocarrier distribution to the subcellular level as well. As with most biological processes, solutions have already been designed by nature and it is often easy to start there.

Viruses could be considered naturally occurring nanocarriers with the ability to deliver their DNA cargo selectively to a subcellular target (the nucleus). It is perhaps safe to say that much of what is known about the cellular interaction and subcellular disposition of nanocarriers has somehow been associated with investigations into mimicking the DNA delivery capability of viruses using artificial nanocarriers [31]. Approaches towards non-viral gene delivery often use perinuclear accumulation as a means of determining the effectiveness of nanocarrier formulation in achieving the nuclear-specific accumulation of the DNA cargo. However, based on their particle size, most nanocarriers are believed to enter the cell by endocytic mechanisms and could therefore be considered as having a predisposition for accumulation in endosomes, and potentially lysosomes as well. It is therefore not unreasonable to assume that it might be possible to control the subcellular disposition of nanocarriers and in so doing to control the disposition of the drug molecules loaded inside them.

At least two major schemes can be imagined to be useful in the design of nanocarriers with the potential for subcellular targeting. The first is based on the inherent predisposition of the nanocarrier for a particular compartment and the second is based on attaching subcellular targeting ligands to the surface of nanocarriers to redirect their accumulation to the desired compartment. Essential to the latter of these approaches is the use of a subcellular targeting ligand. Such ligands could, as in the case of leader sequences, be derived from normal cellular trafficking processes, or, as in the case of triphenyl phosphonium, be based on observations of a predisposition of an organic compound for subcellular compartments. The availability of a wide range of subcellular stains is proof enough that there are several molecules with an inherent ability to accumulate in a particular subcellular compartment. Using the QSAR approach described earlier, it might be possible to identify other candidates for use as subcellular targeting ligands for various intracellular compartments. There is now a growing body of literature exploring subcellular targeted nanocarriers based on these principles.

2.2.1 *Nanocarriers modified with subcellular targeting ligands*

Naturally, any nanoscale drug delivery system intended for the transport of biologically active molecules to subcellular target sites has to avoid lysosomal degradation, as the development of several approaches to achieving endosomal escape would suggest [32,33]. Nevertheless, directing nanomedicine complexes to the endolysosomal system has increasingly gained attention, as pathological conditions associated with endosomes and lysosomes could potentially benefit from therapies targeting these pathways [34]. In the 1970s, enzymes encapsulated in liposomes were proposed as an enzyme replacement therapy for lysosomal storage diseases [35]. More recently, lysosomes and in particular lysosomal hydrolases have been associated with several aspects of malignant transformation, including the loss of cell growth control, altered regulation of cell death, and acquisition of chemoresistance and of metastatic potential [36]. Based on these observations, lysosomes have been proposed as potential target organelles for the chemotherapy of tumors [36]. Furthermore, growing evidence suggests a link between endosomal function in neurons and the etiology of Alzheimer's disease [37]. Endosomal abnormalities have been established invariably to occur within neurons in Alzheimer's disease brains. As endocytic compartments are thought to be involved in the production of the pathogenic β-amyloid peptide, neuronal endosomes have been hypothesized to be the intracellular site of action for inhibitors of β-amyloidogenic APP processing [37].

Precisely targeting particular sites within the endolysosomal network is, however, quite a daunting task. Although endocytosis is a common mechanism almost all cells possess for the internalization of macromolecules, a wide array of such vesicular internalization mechanisms exist [34]. For example, nanoscale drug carrier systems taken up by clathrin-dependent receptor-mediated endocytosis (RME) are most likely to undergo lysosomal degradation, whereas clathrin-independent RME may lead to endosomal accumulation [34]. Consequently, the type of targeting moiety displayed by the nanocarrier system will determine whether the carrier delivers its cargo to either endosomes or lysosomes.

Well-characterized endocytic targeting moieties potentially useful for nanocarrier-mediated drug delivery are folic acid, low-density lipoprotein, cholera toxin B, mannose-6-phosphate, transferrin, riboflavin, the tripeptide RGD, ICAM-1 antibody and nicotinic acid, as recently reviewed in [34]. The cellular internalization mechanisms utilized by these ligands involve clathrin-dependent RME, caveolin-assisted endocytosis, lipid raft-associated endocytosis and cell adhesion molecule (CAM)-directed cellular uptake [34]. However, despite the large variety of known endocytic targeting ligands and the detailed knowledge of their mechanism of uptake, reports about using these ligands for nanocarrier-based delivery of therapeutics to the endolysosomal system to ameliorate pathologies linked to endosomes and lysosomes are rare.

The therapy of lysosomal disorders by means of enzyme replacement may also be hampered by a deficiency of clathrin-mediated endocytosis, as observed in some lysosomal enzyme-deficient cell lines [38-40]. To overcome this obstacle, a new delivery strategy has been proposed that uses nanocarriers targeted to a glycosylation- and clathrin-independent receptor. Intercellular adhesion molecule (ICAM)-1, a glycoprotein expressed on diverse cell types, is upregulated and functionally involved in inflammation, which is a hallmark of many lysosomal disorders [41]. Recombinant human acid sphingomyelinase (ASM) enzyme, deficient in types A and B Niemann-Pick disease, was loaded into nanocarriers coated with anti-ICAM antibody. Anti-ICAM/ASM nanocarriers were found to enter cells by means of CAM-mediated endocytosis, that is, to bypass the clathrin-dependent pathway, and to traffic to lysosomes. The delivered enzyme displayed stable activity and alleviated lysosomal lipid accumulation, suggesting that nanocarriers targeted to ICAM-1 bypassed defunct pathways and could improve the efficacy of enzyme replacement therapy for lysosomal disorders, such as Niemann-Pick disease [41]. A subsequent *in vivo* study determined the impact of carrier geometry on endothelial targeting in the vasculature and on the rate of endocytosis and lysosomal transport within endothelial cells (ECs) [42]. Disks were found to display longer half-lifes in circulation and higher targeting specificity in mice, whereas spheres underwent a more rapid endocytosis. Most interestingly from the aspect of intracellular drug delivery, it was also found that the size of the carrier might determine its intracellular fate. Whereas micrometer-size carriers had prolonged residency in prelysosomal compartments, submicrometer carriers trafficked more readily to lysosomes [42].

Perhaps the most widely used endocytic targeting ligands for the functionalizing of nanoscale drug delivery systems are transferrins (comprehensively reviewed in [43]), a family of large non-heme iron-binding glycoproteins. The efficient cellular uptake of transferrins (Tf) has been and still is being explored for the intracellular delivery of anticancer agents, but also proteins and therapeutic genes. Iron-loaded transferrin binds to a specific cell-surface receptor (TfR1) and on endocytosis via clathrin-coated pits the transferrin–receptor complex is routed into the endosomal compartment, avoiding lysosomal digestion. This is an important feature of TfR1 for drug delivery, as normally glycoproteins taken up by means of receptor-mediated endocytosis are destined eventually to fuse with lysosomes. Such intracellular sorting of endocytosed transferrin from other endocytosed asialoglycoprotein has been found to occur immediately after cell internalization [44]. Following loss of the clathrin coat, the endosome containing the Tf–TfR1 complex then starts taking up protons, which causes the quick acidification of the lysosomal lumen to a pH of ~ 5.5. Recently, a homologue to TfR1 was cloned, called TfR2 [45]. Of importance for anticancer drug delivery, TfR2 was found to be frequently expressed in human cancer cell lines [46]. Encapsulation of doxorubicin into liposomes bearing transferrin on the distal end of liposomal polyethylene glycol (PEG) chains resulted in significantly increased doxorubicin uptake into glioma cells, which are known to overexpress

the transferrin receptor, with the extent of overexpression correlated to the severity of the tumor [47]. Transferrin modification of doxorubicin-loaded palmitoylated glycol chitosan (GCP) vesicles resulted in higher uptake and increased cytotoxicity as compared with GCP doxorubicin alone [48]. Tf vesicles were taken up rapidly with a plateau after 1 – 2 h, and doxorubicin reached the nucleus after 60 – 90 min.

Low-density lipoprotein (LDL) represents another endocytic targeting ligand. Furthermore, LDL itself actually provides a highly versatile natural nanoplatform for the delivery of diagnostic and therapeutic agents to normal and neoplastic cells that overexpress LDL receptors (LDLR) [49,50]. LDL-loading of contrast or therapeutic agents has been achieved by covalent attachment to protein side chains, intercalation into the phospholipid monolayer and extraction and reconstitution of the triglyceride/cholesterol ester core [51]. Zheng *et al*. have constructed a semisynthetic nanoparticle by coating magnetite iron oxide nanoparticles with carboxylated cholesterol and overlaying a monolayer of phospholipid to which apoA-1, apoE or synthetic amphoteric α-helical polypeptides were adsorbed for targeting high-density lipoprotein (HDL), LDL or folate receptors, respectively [52]. These semisynthetic particles have potential utility for the *in situ* loading of magnetite into cells for MRI-monitored cell tracking or gene therapy [51].

Thus far several approaches have been described by which the inherent tendency of nanoparticles to accumulate in the endolysosomal compartment can be exploited or modulated for possible therapeutic purposes. There is also a significant body of work that suggests the feasibility of modifying nanocarriers to redirect delivery of their cargo to other subcellular compartments. Liposomes modified with mitochondriotropic ligands have been shown to improve the efficacy of an anticancer drug both *in vitro* and *in vivo* [53]. To render liposomes mitochondria-specific, the liposomal surface was modified with triphenyl phosphonium (TPP) cations [54]. Methyltriphenylphosphonium cations (MTPP) are rapidly taken up by mitochondria in living cells [55] and have been explored extensively for the delivery of biologically active molecules to and into mitochondria [56-59]. The replacement of the methyl group in MTPP with a stearyl residue was shown to facilitate the attachment of TPP cations to the surface of liposomes [54]. Recently, stearyl triphenyl phoshphonium (STPP) liposomes have been shown to direct effectively the accumulation of rhodamine-labeled phosphatidylethanolamine (Rh-PE) to mitochondria in live cells. A comparison of cell uptake and distribution between STPP liposomes and liposomes prepared from dioleoyl trimethyl ammonium propane (DOTAP) with the same zeta-potential of +30 ± 12 mV suggested that although a positive surface charge of liposomes enhances cell association *per se*, an appropriate organelle-specific ligand on the surface of liposomes is essential for the desired subcellular localization. Subsequent *in vivo* studies were carried out with polyethylene glycol-bearing STPP liposomes. Interestingly, it was found that the cationic TPP ligand did not significantly change the biodistribution of STPP-PEG5000 liposomes in comparison with conventional charge-neutral PEG liposomes. Even more importantly, the tumor accumulation of STPP-PEG liposomes was almost identical to their non-charged counterparts. Finally, with the demonstration of an improvement in *in vitro* and *in vivo* antitumor action of ceramide on incorporation into STPP liposomes, a proof of concept has been established suggesting that the tendency of long-circulating liposomes to accumulate in solid tumors can be combined with organelle-specific tropism conferred by modification with an appropriate ligand to potentiate the effect of an encapsulated antitumor agent. Ceramide in STPP liposomes elicited a strong apoptotic response at ceramide doses as low as 6 mg/kg in comparison with the 36 mg/kg or higher reported with non-targeted liposomes [60].

In addition to liposome and micelles, solid nanoparticles prepared from polymers or colloidal metals also fall under the umbrella of pharmaceutical nanocarriers. Metal nanoparticles, in particular, offer an extra attractive feature, as they are able to convert efficiently near infrared light (NIR) light energy into thermal energy, which makes them attractive agents for thermolysis of cells. Numerous *in vitro* examples have been reported that explore gold nanoparticles (AuNPs) and especially nanorods conjugated to targeting moieties for the thermal ablation of a variety of tumors, as reviewed most recently and comprehensively in [61,62].

Obviously, for heat destruction of a tumor cell, the subcellular disposition of gold nanoparticles is irrelevant and the scarcity of data about their intracellular distribution is therefore not surprising; but intriguing applications of AuNPs for probing and sensing subcellular structures have already been reported. For example, calibrated gold nanoparticles of different sizes have been used to probe the permeability of the mitochondrial outer membrane [63,64]. Rat permeabilized ventricular cells and isolated cardiac mitochondria were incubated under quasi-physiological ionic conditions and during permeability transition with 3 and 6 nm AuNPs, respectively. The authors found that whereas the outer mitochondrial membrane (OMM) was impermeable to 6 nm AuNPS in the absence of permeability transition, the smaller 3 nm AuNPs were able to enter mitochondria. Further incubations of isolated mitochondria with 3 nm particles in the presence of voltage-dependent anion channel (VDAC) inhibitors strongly suggest the VDAC to be the port of entry for the 3 nm particles. In summary, using AuNPs it was possible to determine that the physical diameter of the VDAC is most probably between 3 and 6 nm. Of significant importance from the perspective of subcellular targeting is the finding that the quantity of AuNPs in isolated mitochondria was about 20 times higher than that observed in mitochondria within permeabilized whole cells. The reduced uptake of AuNPs by mitochondria within whole cells in comparison with isolated organelles is not surprising owing to the lack of surface-linked mitochondria-specific targeting ligands. However, gold nanoparticles are a flexible nanoscale platform for the conjugation of a variety of targeting ligands based on the affinity of thiol

and amino groups for the gold surface. Of particular interest here is the report of the conjugation of the triphenyl phosphonium mitochondriotropic ligand [29] to the surface of AuNPs [65]. Triphenyl-phosphonioalkylthiosulfate and potassium tetrachloroaurate were dissolved in dichloromethane followed by drop-wise addition of an aqueous solution of sodium borohydride to generate 5 – 10-nm-sized AuNPs with surface-attached triphenylphosphonium residues. Although data describing the intracellular localization of these potentially mitochondriotropic AuNPs have not yet been made available, AuNPs have already been targeted to the nucleus using the adenoviral nuclear localization signal (NLS) and integrin binding domain [66]. Such an approach has been reported to be useful in the development of probes for cell tracking by surface-enhanced Raman scattering. Gold nanoparticles were surface functionalized with the SV40 NLS, which led to accumulation of the nanoparticles in the nucleus of HeLa cells [67].

The potential of leader sequence peptides to overcome intracellular barriers to DNA delivery was first demonstrated a decade ago [68]. To facilitate the import of exogenous DNA into the nucleus, a capped 3.3-kbp CMVLuciferase-NLS gene containing a single nuclear localization signal peptide (PKKKRKVEDPYC) was synthesized. The resulting transfection enhancement due to the nuclear leader peptide was ~ 10 – 1000-fold, irrespective of the cationic vector or the cell type used. At that time the authors hypothesized that the 3-nm-wide DNA present in the cytoplasm was initially docked to and translocated through a nuclear pore by the nuclear import machinery and as DNA entered the nucleus it was quickly condensed into a chromatin-like structure, which provided a mechanism for threading the remaining worm-like molecule through the pore [68].

Taking the concept further was a proposal for an 'ideal' nuclear non-viral polycation-based transfection vector based on the incorporation of nuclear leader sequence peptides into receptor-targeted polyplexes containing cell-targeting ligands (such as transferrin or epidermal growth factor) with PEG serving the dual functions of shielding agent and endosomolytic agent [69]. A series of subsequent papers explored the potential of integrating most of these functions into a variety of polyplexes [70-73].

Modification with a leader sequence peptide has also been applied to creating delivery systems for mitochondria. A mitochondrial leader peptide (MLP), derived from the nucleocytosol-expressed but mitochondria-localized ornithine transcarbamylase, was recently used to render polyethylenimine (PEI) mitochondriotropic [74]. PEI had been developed in the mid-1990s as a versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo* [75,76]. Lee *et al.* [74] conjugated the mitochondrial leader peptide to PEI by means of a disulfide bond and confirmed the complex formation of PEI–MLP with DNA by a gel retardation assay. *In vitro* delivery tests of rhodamine-labeled DNA into living cells demonstrated that PEI–MLP/DNA complexes were localized at mitochondrial sites, in contrast to controls carried out with PEI–DNA complexes lacking MLP. The authors' data suggest that PEI–MLP can deliver DNA to the mitochondrial sites and may be useful for the development of direct mitochondrial gene therapy, a strategy for the cure of mitochondrial DNA diseases proposed earlier [77-80] as an alternative to allotropic expression [81-84].

2.2.2 *Nanocarriers prepared from self-assembling molecules with known subcellular accumulation*

All the examples discussed in the previous section share a common assumption that unless a targeting ligand is incorporated into the design, the nanocarriers will remain in the endolysosomal compartment. However, it is interesting also to consider the disposition of a nanocarrier made exclusively of a molecule with a predisposition for a subcellular compartment. A good example of a molecule that has a strong affinity for a subcellular compartment and that is also capable of self-assembling to form a potential carrier system is the mitochondriotropic amphiphile dequalinium chloride. A serendipitous discovery while screening mitochondriotropic drugs potentially able to interfere with the mitochondrial DNA metabolism in *Plasmodium falciparum* [85] revealed this self-associating tendency of dequalinium chloride and its ability to form vesicles. At the time of their discovery, these unusual vesicles were termed DQAsomes (pronounced dequasomes), that is, dequalinium (DQA)-based liposome-like vesicles [86]. Based on the fact that these carriers are composed exclusively of mitochondriotropic molecules and that they are able to bind and protect DNA, DQAsomes were explored as potential mitochondria-specific DNA delivery vehicles for direct mitochondrial gene therapy [87-90]. More recently, DQAsomes have also been explored as a mitochondria-targeted nanocarrier system for small drug molecules, in particular for anticancer drugs known to trigger apoptosis through direct action on mitochondria [91,92]. For these studies, paclitaxel was used as a model drug.

Paclitaxel is a well-known antitumor agent used in the treatment of several cancers. Clinically, the therapeutic potential of paclitaxel is limited owing to a very narrow span between the maximal tolerated dose and intolerable toxic level. In addition, its poor aqueous solubility requires the use of emulsion formulations containing Cremophor EL, a surfactant of considerable toxicity in itself [93]. Paclitaxel is largely believed to exert its action by stabilizing the microtubules of cells, but the precise mechanism of paclitaxel-induced apoptosis remains unclear [94,95]. It has also become evident that paclitaxel has other targets inside the cell, most notable of which is the mitochondrial network. For example, it has been shown that paclitaxel can act directly on isolated mitochondria from human neuroblastoma cells to induce the permeability transition pore (PTP)-dependent release of mitochondrial cytochrome *c* [96].

Very interestingly from the perspective of subcellular drug targeting, a 24 h delay has been observed between the

paclitaxel-triggered release of cytochrome *c* in intact cells versus the cell-free system [97]. This delay has been attributed to the existence of several drug targets inside the cell, making only a subset of the drug molecules available for mitochondria [97]. Hence, paclitaxel appears to be a molecule whose action may be significantly improved by specific subcellular delivery to the mitochondrion. It was shown that DQAsomal encapsulation of paclitaxel improved *in vivo* efficacy against human colon cancer tumors in nude mice [91]. Recently it was confirmed that DQAsomal encapsulation changes the subcellular distribution of a labeled derivative of paclitaxel [92]. Confocal fluorescence microscopic images demonstrate that in contrast to free paclitaxel, the DQAsomal-encapsulated drug at least partially colocalizes with mitochondria [92]. Subsequently, the metabolic cytotoxicity was compared with the apoptotic activity. As anticipated, it was found that although encapsulation of the drug into DQAsomes did not significantly alter the dose-dependent cytotoxicity of paclitaxel, it appeared to improve the proapoptotic action [92], as determined by a quantitative nuclear morphology assay and a DNA fragmentation assay. DNA fragmentation data also indicated that 10 nM DQAsomal-encapsulated paclitaxel was comparable to 50 nM free paclitaxel in inducing DNA fragmentation characteristic of apoptosis, that is, DQAsomal encapsulation increased the apoptotic activity of paclitaxel ~ 5 times [92].

The antitumor efficiency of DQAsomal-encapsulated paclitaxel was most recently enhanced further by modifying the DQAsomal surface with folic acid (FA) [98]. The folate receptor is a folate high-affinity membrane-binding protein that is overexpressed in a large variety of human tumors [99-101]. Folic acid conjugates are internalized in a tumor cell-specific manner by receptor-mediated endocytosis, resulting in an increased toxicity of the corresponding drug [102-104]. Incubation of EDC-activated FA-PEG-COOH with preformed paclitaxel-loaded DQAsomes at different molar ratios resulted in DQAsomes in which ~ 5.3% of all dequalinium molecules were conjugated to FA-PEG-COOH [98]. Cell cytotoxicity studies using folate receptor-expressing HeLa cells suggested that folic acid-conjugated DQAsomes possess better antitumor activity as compared with plain paclitaxel-loaded DQAsomes, folic acid-conjugated paclitaxel-loaded liposomes and the free drug. Based on the data, it was concluded that folic acid-conjugated DQAsomes delivered the drug not only to the cytosol but also to mitochondria, whereas folic acid-conjugated liposomes delivered the drug into the cytosol only [98].

Although DQAsomes are still far from being the perfect delivery system, some basic proof of concept for an alternative strategy towards the design of subcellular targeting nanocarriers seems to have been established. It is also obvious that to design similar carriers for other subcellular compartments it would be necessary first to find self-assembling molecules with an affinity for the intended subcellular compartment. To this end, recent work on the subcellular distribution of micelle-forming agents offers some interesting insights [105-109].

Micelles are nanoassemblies of amphiphilic molecules in which the hydrophobic parts of the amphiphiles form the water-excluding inner core, whereas the hydrophilic ends of the amphiphiles make up the corona or shell. The use of micelles for the delivery of hydrophobic drugs was introduced in the early 1980s [110] and was greatly extended during the 1990s by the development of block copolymer micelle-based drug delivery vehicles, leading to the first clinical trials conducted in the early 2000s (reviewed in [111]). Imaging studies based on the use of a variety of organelle-specific dyes, gold and fluorescent polymers have provided detailed insight into the subcellular distribution of block copolymer micelles [106,112]. Both imaging techniques, that is, confocal fluorescence microscopy (to detect the fluorophore-labeled copolymers) and transmission electron microscopy (to detect the gold-labeled copolymers), demonstrate that poly(caprolactone)-*b*-poly(ethylene oxide) micelles (PCL-*b*-PEO micelles) do not enter the nucleus. With respect to the cytosolic distribution of PCL-*b*-PEO micelles, however, the two different imaging techniques used in these studies suggest quite a different subcellular disposition. Transmission electron microscopy (TEM) images show most of the gold-labeled micelles to be localized in endosomes/lysosomes and a few of them were seen at or in mitochondria [106]. Confocal fluorescence microscopic images, on the other hand, show fluorescent PCL-*b*-PEO micelles almost evenly distributed throughout the cytosol [112]. Therefore, not surprisingly, cell staining with organelle-specific dyes and overlaying the corresponding confocal fluorescence images reveal partial colocalization of PCL-*b*-PEO micelles with lysosomes, with the Golgi apparatus and the endoplasmic reticulum, with the mitochondria and the endoplasmic reticulum and with mitochondria alone. Considering the nature of the micelle corona, which is entirely made up of non-functionalized polyethylene oxide, a highly hydrophilic polymer, any specific interaction with or any specific affinity for any of the cell organelles could not be expected *per se*. It would be very interesting to see to what extent modifying the micelle corona with organelle-specific ligands would alter the intracellular distribution of such micelles, which then potentially could become nanocontainers that distribute cargo to defined cytoplasmic organelles. However, the distinctive distribution of non-functionalized PCL-*b*-PEO micelles throughout the cytosol makes them highly suitable for multiple cytoplasmic targeting [112], which has recently been proved to be relevant for the delivery of effector molecules of the cell signaling pathways that are activated in the cytosol. Savic *et al.* [108] loaded PCL-*b*-PEO micelles with c-Jun NH(2)-terminal kinase inhibitor SP600125 in order to explore their potential to rescue isolated human islets of Langerhans. To investigate the effectiveness of micelle-incorporated SP600125 in preventing islet cell death, the islets were challenged with TNF-α, IL-1 and IFN-γ. It was shown that micelle-incorporated SP600125 did not lose its inhibitory activity during incorporation into micelles, and it protected the islets against cytokine-induced

loss of viability to the same extent as control SP600125. Most importantly, the concentration of micelle-incorporated SP600125 used was 13-fold lower, demonstrating the greater efficacy of micelle-delivered SP600125. This study suggests that micelle-based intracellular delivery of potent, poorly water-soluble cell-death-pathway inhibitors may represent a useful addition to established delivery of cytocidal block-copolymer micelle-incorporated bioactives [108].

2.3 Improving understanding of subcellular transport and nanoparticle trafficking

Based on the examples discussed so far it would seem that there is indeed hope that nanocarrier systems could be designed to achieve true molecular-level targeting inside cells. However, to say that these systems will be available soon is perhaps premature given what little is known about the subcellular dynamics associated with nanoparticle trafficking. There are, in the authors' opinion, several unanswered questions. For example, do all nanocarriers remain intact on cell entry and subsequent disposition? Are there differences in the disposition of vesicles in comparison with particles? What is the true influence of size on the intracellular disposition of various nanocarriers? Most important, however, is the question of the mechanism by which the nanocarrier is able to achieve selective uptake and delivery into the subcellular compartment. All the strategies described so far report observations of altered or improved subcellular accumulation that appears to result in improved activity, but how exactly this happens is still unclear. Do the nanocarriers remain intact on internalization and are then trafficked as intact structures? If so, how is the therapeutic cargo released to the correct subcellular compartment? Alternatively, it could be imagined that once taken up into the early endosomal vesicle, the nanocarrier components undergo a redistribution to become part of the endosomal vesicle. There is some evidence to suggest that in fact cells actively traffic nanocarriers in cell membrane-derived vesicles [113]. Assuming the targeting ligand was able to redistribute to the surface of the endosomal vesicle, it might be possible that the vesicle would have an altered subcellular fate that could involve transport to and association with a target compartment other than the lysosome. Although this may seem to be far-fetched speculation, there has already been some work along similar lines towards the development of nanocarrier systems for delivery of molecules to the nucleus and even the mitochondria. Based on the premise that to deliver DNA efficiently to the nucleus a delivery system must penetrate through the plasma membrane, nuclear envelope, before DNA release in the nucleus, a strategy that involved step-wise membrane fusion was devised. Using a multilayered nanoparticle called a tetra-lamellar multifunctional envelope-type nano device (T-MEND) and consisting of a DNA-polycation condensed core coated with two nuclear membrane-fusogenic inner envelopes and two endosome-fusogenic outer envelopes, which are shed in stepwise fashion, transgene expression in non-dividing cells was reported to be dramatically increased [114]. A similar approach in designing a mitochondria-specific delivery system has been reported as well. Liposomal carriers called MITOporters, which carry octaarginine surface modifications to stimulate their entry into cells as intact vesicles (via macropinocytosis), were prepared with lipid compositions that were identified in various experiments to promote both fusion with the mitochondrial membrane and the release of liposomal cargo to the intramitochondrial compartment in living cells. Using GFP protein as a model cargo, it was shown that MITOporter liposomes are able to deliver their cargo to mitochondria selectively [115,116].

It is also interesting to note that changes in nanoparticle architecture result in changes in subcellular disposition [117]. Fluorescein isothiocyanate-labeled layered double-hydroxide (LDH) nanoparticles were prepared from Mg_2Al under conditions that yielded either hexagonal sheets (50 – 150 nm wide and 10 – 20 nm thick) or nanorods (30 – 60 nm wide and 100 – 200 nm long). A comparison of the subcellular distribution of these two types of preparation revealed that the nanorods trafficked to the nucleus but the hexagonal sheets remained in the cytoplasm [117]. Not surprisingly, an active microtubule-mediated transport process is hypothesized to be responsible for the observed rapid nuclear accumulation of the nanorods [117].

3. Conclusions

Various nanocarrier platforms have already undergone preliminary investigation for their ability to control the subcellular distribution of drug molecules. Several small organic molecules as well as peptide sequences can be used to surface modify nanocarriers to control their intracellular fate. In addition, physical attributes of the nanocarrier such as particle size and aspect ratio have been shown to affect the subcellular distribution of the nanocarrier. Most importantly, several examples serve to provide preliminary proof of concept that nanocarrier-mediated selective delivery of a drug to its subcellular site of action can improve its action both *in vitro* and *in vivo*.

4. Expert opinion

As illustrated in this review, there are several innovative strategies now under investigation that hold the promise of improved therapy through the use of nanocarriers for subcellular targeting of bioactive molecules. A common paradigm that seems to apply to most of these approaches is the use of subcellular targeting ligands to control the subcellular distribution of nanocarriers. Given the relative ease of modifying nanocarriers with various surface functionalities and the fact that such approaches are already in use to achieve targeting at a cellular and organ level, the ligand-based approach does seem to be a logical extension of current technology. It helps that new tools are concurrently being investigated to understand some of the physicochemical aspects of how

small molecules [28,29] as well as proteins [118] are able to accumulate selectively in certain subcellular compartments to afford the rational design of a wide repertoire of subcellular targeting ligands. However, we cannot help but be cautious in the knowledge that the approaches described so far have yet to be tested extensively *in vivo* and are based only on current understanding of subcellular trafficking. What little is known of subcellular trafficking processes is based largely on studies with solid nanoparticles and on quantum dots [119-125] Whether these observations can be extended to vesicular carriers such as liposomes and micelles remains a question, and in the authors' opinion is due in large part to current limitations in imaging technology. The authors are, however, hopeful that with technological advances in real time fluorescence confocal imaging of live cells [126-129] as well as the emergence of new imaging techniques such as total internal reflection microscopy [130] and label-free approaches such as Raman microscopy [131-133], some of the questions posed in previous sections might be more satisfactorily answered. It also must be considered that the need for subcellular targeting might depend as much on the subcellular target as on the drug molecule. A subcellular targeting carrier can be imagined to be useful only in those circumstances where the unaided drug does not accumulate in sufficient quantities to saturate completely all available target sites. It is also obvious that the delivery system should not present its own toxicity issues. With the emergence of nanotechnology applied to numerous areas of research and development, including manufacturing of common goods, agriculture, industry, business and public health and medicine, nanotoxicity research has materialized as a whole new subdiscipline of toxicology [134,135]. Nanomaterials are distinguished from bulk materials of the same chemical composition as well as from their corresponding atomic or molecular components by different chemical, physical and biological properties. With the exception of nano-objects made up entirely of biodegradable components such as liposomes, the interaction of nano-specific characteristics with biological systems (i.e., the environment as a whole as well as individual living organisms) is still poorly understood. The evaluation of the safety of medicinal nanomaterials, in particular for long-term applications, is an important challenge for the near future [136]. At present, any particular biological response to any specific characteristic of the nanomaterial cannot be predicted because of the lack of reliable databases [136]. Subsequently, a case-by-case approach for the identification of potential hazards has to be followed [136]. At the same time, standard references and methodology for the risk assessment of nanomaterials need to be developed [137]. In summary it would appear that although nanocarriers represent a flexible platform to achieve targeting of bioactive molecules to cell organelles, several hurdles must be crossed before this technology can truly be translated to use in the clinic.

Declaration of interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

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