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Controlling subcellular delivery to optimize therapeutic effect

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Abstract

This article focuses on drug targeting to specific cellular organelles for therapeutic purposes. Drugs can be delivered to all major organelles of the cell (cytosol, endosome/lysosome, nucleus, nucleolus, mitochondria, endoplasmic reticulum, Golgi apparatus, peroxisomes and proteasomes) where they exert specific effects in those particular subcellular compartments. Delivery can be achieved by chemical (e.g., polymeric) or biological (e.g., signal sequences) means. Unidirectional targeting to individual organelles has proven to be immensely successful for drug therapy. Newer technologies that accommodate multiple signals (e.g., protein switch and virus-like delivery systems) mimic nature and allow for a more sophisticated approach to drug delivery. Harnessing different methods of targeting multiple organelles in a cell will lead to better drug delivery and improvements in disease therapy.

The 1975 discovery of the '**signal hypothesis**' led Gunter Blobel to win a Nobel Prize [1–3]. Blobel predicted that 'zip codes' were responsible for targeting certain proteins to subcellular compartments such as the cytoplasm, nucleus, nucleolus, mitochondria, endoplasmic reticulum (ER), Golgi and peroxisomes (Figure 1). The intracellular delivery of a pharmaceutical agent can have a dramatic impact on its therapeutic efficacy. Indeed, precise compartmentalization of certain drugs is necessary for their biological effect. For example, agents intended for gene therapy must be eventually delivered to the nucleus in order for the therapeutic protein to be expressed. Some drugs, such as RNAi, must target the cytosol in order to block the cells' mRNA. In other cases, pro-apoptotic drugs can be selectively targeted to the mitochondria where they exert their actions.

Our previous work has shown that an oncogene can be targeted to a different cellular compartment to completely alter its function. The causative agent of chronic myelogenous leukemia (CML), Bcr-Abl, is normally found in the cytoplasm where it acts as an oncogene. However, when targeted to the nucleus (by attaching four nuclear localization signals [NLSs]) it acts as an apoptotic factor [4]. In this case, targeting a protein to a single organelle can be used to elicit a desired effect such as apoptosis. Further work in our laboratory focuses on controlled localization of proteins to alter function. We have described our 'protein switch' technology that allows controlled translocation from the cytoplasm to the nucleus upon addition of a ligand [5–7]. One of the main goals of our research is to imitate the function of proteins found in nature. Many proteins in signal transduction pathways are localized to one

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compartment initially, bind to protein partners in the cell (or are signaled by other proteins) and change their location in the cell, leading to an alteration in function. Ultimately we seek not only targeting of a specific organelle, but also a further level of sophistication, where multiple signals can be used to target multiple organelles. Indeed, the ability to precisely target drugs to different organelles is changing the way therapeutics are developed. This article will focus on therapeutic targeting to specific cellular organelles (Figure 1). Each organelle will be briefly described, followed by methods to reach and target the organelle for therapy. Finally, future perspectives for therapeutic delivery will be discussed.

Cytosolic delivery

Typically, drugs targeting cellular organelles have to be initially delivered to the cytosol, which in some cases is the site of action, where drugs (e.g., glucocorticoids, proteins or siRNA) bind to their receptors or act on other targets. There are three main barriers that must be overcome to facilitate cytosolic delivery:

- Evading detection by the reticuloendothelial system (RES)
- Interaction with the cell membrane and internalization
- Intracellular trafficking and endosomal escape

Evading RES detection

In order for cytosolic delivery to occur when the drug is delivered into the bloodstream, the drug first has to evade detection by the RES prior to interacting with cell membranes. The endothelial layers of the liver, spleen and bone marrow comprising the RES contain mononuclear macrophages that filter the blood of foreign pathogenic particles [8]. Aggregation of foreign particles in the presence of plasma proteins and cell adhesion molecules facilitates rapid clearance from the bloodstream [9–12]. To lengthen the circulatory time and decrease macrophage detection, several approaches have been attempted. Liposomes have been one of the most common drug delivery agents used to evade RES detection. Regular liposomes undergo rapid opsonization via the RES cells, followed by lysosomal degradation. Strategies to evade RES detection have included use of targeted liposomes (for accumulation in target organs) and/or using **'stealth' liposomes** [13]. Long-circulating liposomes (stealth) can be prepared by including amphiphilic stabilizers (e.g., cholesterol) [14–16], phosphatidylinositol and gangliosides [17], or a hydrophilic surface by grafting with polyethylene glycol (PEG) [18]. The combination of long-circulating and targeted liposomes has been extremely popular in the last decade (e.g., antibody targeting and PEG) [13].

Cell membrane interaction

Subsequently, therapeutics interact with cell membranes (plasma membranes), which are lipid bilayers composed of phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin are common phospholipids), cholesterol, glycolipids and proteins (including proteoglycans, which contain a core protein with one or more glycosaminoglycans attached) [19]. Proteoglycans are important for delivery of drugs via cationic polymers [20,21]. For example, an ionic interaction occurs between cationic polyethyleneimine (PEI) and the cell membrane, which has negatively charged sulfated proteoglycans on the cell surface [20].

Conjugation with cell-penetrating peptides can also overcome the barrier of the cell membrane [22,23]. In the late 1980s and early 1990s many groups ([24–26] and others) found that a protein transduction domain of 9–16 cationic (polyarginine like) amino acid residues had cell membrane-penetrating capability, including the ability to cross the nucleus. Several other groups [27] showed that peptides and proteins attached to cell-penetrating peptides can cross

cell membranes (reviewed in [28]). Many therapeutics have been delivered to cells in this manner [29–33].

Internalization may occur by endocytosis (initiated by electrostatic or hydrophobic interactions with the cell membrane, or interaction with a cell-surface receptor) followed by endosomal escape, or by other means, such as macropinocytosis, or combinations of these [13]. After trafficking into the cytosol, the drug may either exert its action or traverse to a subcellular compartment (e.g., nucleus, mitochondria and peroxisome). For example, for delivery of genes, the cytoplasmic barrier is only the first step before delivery to the nucleus can occur. Table 1 depicts various agents designed to target the cytosol and their corresponding cargo (drug).

Liposomes, cationic lipid DNA and polymer complexes have been used as nonviral delivery carriers by complexing with DNA [34,35]. Nanoparticles can encapsulate various types of therapeutics including low-molecular-weight drugs [36,37] and macromolecules (e.g., proteins and DNA). Liposomes bind to cell membranes and are internalized via endocytosis with pH-sensitive liposomes being more efficient [38,39]. The formulation of pH-sensitive liposomes with phosphatidylethanolamine increases affinity to adhere to cell membranes due to the poor hydration of its head groups leading to aggregate formation [40,41]. Other liposome examples include thermosensitive liposomes, which allow temperature-sensitive release of drug [42–50]. Development of drug carriers is still in progress to overcome cytosolic barriers, including biodegradable polymeric carriers for controlled release [51,52].

Conjugating antibodies to either liposomes or polymers increases internalization via a receptor-mediated endocytotic process [53,54]. Others have targeted ligands to their respective cell-surface receptors (folate receptor [55], transferrin receptor [56], low-density lipoprotein receptor [57] and many other ligand-receptor-mediated methods [58]) for endocytosis and eventual release into the cytoplasm.

It may be desirable to retain a protein or peptide, once inside a cell, in the cytoplasm, where it may interact with its target (e.g., a signal transduction protein or receptor). Proteins smaller than approximately 45 kDa can passively diffuse into the nucleus; exclusion from the nucleus (hence cytoplasmic localization) can also be conferred by increasing the size of the gene therapy protein to over 60 kDa [59]. Such large proteins cannot traverse the nuclear pore complex (NPC) passively, and require NLSs for entry into the nucleus via the NPC. Proteins, peptides and DNA may be tagged with amino acid sequences that confer nuclear export (using a nuclear export signal [NES]) to achieve this goal, or in the case of a gene, the NES may be encoded by plasmid DNA and genetically engineered to the therapeutic gene. Our laboratory uses the latter to confer cytoplasmic localization to engineered proteins (Table 1) [5–7,60]. NESs are composed of sequences that are leucine rich, approximately 10–12 amino acids in length. Proteins with NESs are exported out of the nucleus by the classical export receptor, CRM-1 or exportin-1 (Figure 2A) [59]. A common consensus NES is LX (1–3) LX (2–3) LXJ where L is leucine, X is a spacer (numbers in parenthesis indicate number of amino acids in the spacer) and J is leucine, valine or isoleucine [6,7,60].

The endosome/lysosome

Endosomal/lysosomal barriers

Drugs that have entered cells via endocytosis (and need to be delivered to the cytosol) are faced with a third obstacle: late endosomal/lysosomal degradation. The process of endocytosis starts at the cell surface. The material to be internalized gets encapsulated by a small portion of the plasma membrane (clathrin-coated pit formation), followed by a pinching off to form an endocytic vesicle. In the case of pinocytosis, some vesicles may originate at caveolae (instead of clathrin). Receptor-mediated endocytosis is a common mechanism for endocytosis of drugs/

drug carriers. Endocytosed material that is not retrieved from endosomes (in the case of recycled receptors, for example) is destined for the lysosome [19]. Lysosomes contain hydrolytic enzymes for digestion of phagocytosed materials, and macromolecules (derived intra- or extra-cellular), and even for production of nutrients [19].

The classic example uses PEI for endosomal escape [61]. PEI's secondary and tertiary amines are protonated in the acidic environment of the endosomes. The '**proton sponge effect**' causes osmotic swelling and rupture of the endosomes [62]. The main drawback of PEI is its toxicity [63,64], but many modifications of PEI have been made in an attempt to circumvent this [65–69].

Many other techniques to escape the endosome (hence bypassing degradation) have been investigated. These include the development of pH-sensitive liposomes and polymers, where they switch from a membrane-inert (pH 7.4) to a membrane-disruptive (under acidic conditions) conformation [70]. This facilitates a destabilization of the endosomal membranes, resulting in release of the encapsulated therapeutic in the cytoplasm (reviewed in [37]). Furthermore, membrane-disruptive polymers masked by PEG via disulfide groups and acid-degradable acetal groups disrupt the endosomal membrane when the acidic pH of the endosome degrades the acetal groups unmasking the membrane-disruptive backbone [71]. Subsequently, the disulfide groups are reduced in the cytoplasm releasing the conjugated drug. Another strategy is to use membrane-disruptive peptides mimicking the viral mechanism of endosomal escape. These peptides contain acidic residues that prevent the formation of an α-helix when unprotonated, but under slightly acidic pH the peptide forms an α-helix that allows multi-merization and protein interaction, leading to endosomal escape.

Endosomal/lysosomal targeting

A drug may also be targeted to the endosome/lysosome directly for a therapeutic effect. There are over 40 lysosomal storage diseases that occur as a result of lysosomal enzyme deficiency, which allow various proteins, lipids and carbohydrates to accumulate in the lysosomes. These diseases usually manifest as neurodegenerative, but can be treated with enzyme-replacement therapy (reviewed in [72]). Drug delivery to endosomes has been achieved by targeting cell-surface receptors (mannose-6 receptor, reviewed in [73] and the IGF-II/cation-independent mannose 6-phosphate receptor [74]) for receptor-mediated endocytosis.

Nucleus

Transport through the NPC

Nucleocytoplasmic exchange occurs via pores formed by NPCs embedded in the nuclear envelope [75]. The 125-MDa NPC is the gatekeeper for nuclear entry, and the central role of this complex is confirmed by the high level of conservation among all eukaryotes [76–78]. This complex regulates all trafficking into and out of the nucleus, including passive diffusion of molecules smaller than 9 nm in diameter (<40 kDa) and active transport of molecules between 9 and 40 nm in diameter (40 kDa to 60 MDa), and has an estimated ability to translocate 1000 molecules per second [79–81]. Cytoplasmic filaments, a central channel and a nuclear basket give the NPC a tripartite structure constructed from approximately 100 nucleoporins (**Nups**) [82–84] (reviewed in [5]). The long cytoplasmic filaments project into the cytoplasm and interact with karyopherins (family of transport receptors) and deflect nonshuttling proteins [85–88]. The central channel is a cylindrical intramembrane transporter (central aqueous channel) with a 9 nm diameter pore [89–91] and is anchored to the nuclear membrane via eight spokes. The nuclear basket consists of eight long filaments projecting into the nucleoplasm, which are connected distally by a ring.

While molecules that are small enough to fit through the 9-nm pore in the central channel can enter the nucleus passively, larger molecules must be escorted through the NPC by karyopherins. Karyopherins, such as importin α, recognize and bind specific amino acid sequences termed NLSs [59,92–95]. Classical NLSs are monopartite with a single stretch of amino acids similar to the NLS found in SV-40 large tumor antigen (PKKKRKV; critical residues underlined) [96], or a consensus of K(R/K)X(R/K) [97] (Figure 2B). Bipartite NLSs (two short amino acid sequences with a spacer in between) have also been identified in proteins such as nucleoplasmin (KRPAATKKAGQAKKKKLDK) [98]. Classical NLS sequences are recognized by importin α [99,100], which binds to the HEAT repeats (helical repeats of histidine, glutamic acid, alanine and threonine) of importin β [101] through an importin β binding domain [102]. Once this ternary complex is formed, importin β mediates the translocation of the complex into the nucleus through interactions with FG (F: Phe; G: Gly) amino acid repeats of Nups in the NPC [103]. Inside the nucleus the complex dissociates when RanGTP binds to importin β [104], and both importin α and importin β are escorted separately back to the cytoplasm by RanGTP [100,105,106]. In the cytoplasm, RanGAP catalyzes the conversion of RanGTP to RanGDP, and importin α and importin β are ready for another cycle of import.

Challenges of nuclear trafficking

It is not a simple task to diffuse through the highly viscous cytosolic fluid. DNA over 2 kb is almost immobile in the cytosol, and DNA over 250 bp has reduced lateral mobility [107, 108]. Nevertheless, a mechanism to overcome the challenge of diffusing through the cytosol is demonstrated by viruses that use active transport along the microtubule cytoskeleton [109–112]. Another challenge in nuclear targeting is the translocation through the nuclear envelope, which has been described as one of the limiting steps for nonviral gene delivery [113]. The midplane of the pore in the NPC is the narrowest and is estimated to be approximately 40 nm in diameter [114]. Thus, the largest molecule able to fit through this pore and enter the nucleus is roughly 40 nm, an estimate that has been confirmed (39 nm) using gold nanoparticles coated with NLSs [115]. The 36-nm capsid from hepatitis B can also transport through the NPC into the nucleus without dissociating [115], further demonstrating the capabilities of the NPC and validating the 40-nm size limitation. However, plasmid DNA in complex with polylysine crosslinked with NLSs, 60 nm in diameter, has been shown to enter the nucleus [116,117], likely due to the increased flex-ibility of this complex versus the more rigid gold nanoparticles. Molecules larger than 40–60 nm in diameter, regardless of containing a NLS, will be unable to enter the nucleus [116,117].

Methods of NLS incorporation

Various methods of incorporating a NLS into the therapeutic have been attempted (reviewed in [113,118]). For peptide/protein therapeutics, genetic engineering provides a facile method of incorporating the NLS as we have recently demonstrated with the oncoprotein Bcr-Abl, which causes CML [4]. However, for nonpeptide/protein therapeutics, the attachment of the NLS is no trivial matter and can result in drastically different results. As gene therapy is one of the most extensively researched areas in nuclear targeting, Table 2 provides examples of various methods of linking NLSs to DNA for gene therapy.

Of these approaches, electrostatic interactions are the simplest, but are not without drawbacks. One potential problem is the dissociation of the complex in the cytosol, leaving the DNA without the nuclear targeting signal. Furthermore, as most electrostatic interactions with DNA are not sequence specific, the interactions may interfere with the transcription of the gene after being delivered to the nucleus. To circumvent this problem, peptide nucleic acid with a NLS has been used to interact with the DNA in a sequence-specific manner in a region other than the gene of interest [119], but only achieved modest improvements. Numerous attempts to

improve efficiency through covalent attachment of the NLS have been attempted, but many have failed to demonstrate improved nuclear translocation and gene expression [120–123]. However, initial attempts to covalently attach the NLS to the DNA did not control where on the DNA strand the NLS was attached and may have resulted in the NLS preventing the efficient transcription of the gene of interest.

Further attempts were then made to attach the NLS to DNA upstream or downstream from the gene of interest to avoid interference with transcription, but did not achieve the anticipated results. The triple helix with photoactivation attempted by Neves *et al.* [124], the conjugation to the hairpin attempted by Tanimoto *et al.* [125] and linear DNA amplified via PCR with NLS-conjugated primers [126,127] all demonstrated a lack of transfection efficiency, and even a lack of nuclear tanslocation. However, one study has shown that covalent attachment of a NLS to a hairpin at the 3′-end of linear DNA can enhance transfection anywhere from 10- to 1000-fold depending on the cell type [128], but similar schemes did not achieve the same result by others.

Therapeutics targeting the nucleus

Pharmacological agents that alter the nuclear translocation (either inhibit or induce translocation) also provides a means for therapeutic intervention [129]. Inhibition of the NPC through binding FG amino acid repeats by antibodies has been used to prevent nuclear accumulation [130] but is not specific and inhibits all protein translocation into the nucleus. These antibodies, RL2 and mAb414, are further limited by complications with delivery of an antibody inside a cell. Wheat germ agglutinin is a lectin that binds to *N*-acetylglucosamine common to certain Nups and also inhibits general nuclear transport through the NPC [131, 132]. Kosugi *et al.* have also demonstrated that nuclear translocation can be inhibited through two peptides, bimax1 and bimax2, that bind to importin α [133]. The inhibition of the NPC reduces nuclear transport and results in reduced nuclear localization, while the inhibition of nuclear export can increase the nuclear accumulation of proteins. Leptomycin B (LMB) is a general nuclear export inhibitor that binds to CRM-1 [134–136], a protein that escorts other proteins with NESs from the nucleus to the cytoplasm. Although LMB has antitumor properties, it was also found to be highly toxic and cannot be used clinically. Recently, Mutka *et al.* have identified LMB analogs that retain the potency of LMB, are better tolerated and have shown potential as cancer therapeutics in mouse xenograft models [137]. Still, inhibiting the general nuclear import or export of all proteins may be limited as a therapeutic approach and methods to target specific proteins would be a better alternative.

An example of therapeutic intervention through altering the nuclear localization of a specific protein is the use of peptides that bind nuclear factor-κB (NF-κB) and prevent its nuclear translocation [138,139]. The misregulation of the transcription factor NF-κB has been associated with cancer and autoimmune diseases, both of which potentially could be treated by inhibiting the nuclear accumulation of NF-κB [140]. A similar approach of inhibiting nuclear translocation through binding with a peptide has been demonstrated for nuclear factor of activated T cells [141]. Moving proteins from the cytoplasm into the nucleus has also been demonstrated through the use of **protein aptamers** containing a NLS [142]. We have also developed a sophisticated chimeric protein we have termed a protein switch (Figure 3), for controlled nuclear transport [6,7,60]. This protein switch (containing both a NES and an inducible NLS) localizes to the cytoplasm, but upon the addition of a ligand, it translocates into the nucleus. This controlled nuclear translocation can be used to alter nuclear localization of endogenous proteins; attempts to do so are currently underway in our laboratory.

Targeting the nucleolus

A subcellular compartment such as the nucleolus presents additional challenges in identifying specific targeting sequences as it exists in a highly dynamic equilibrium with the nucleoplasm and is not enclosed by a membrane. The nucleolus has been described as a machine for ribosome production and is a dense area composed of nucleolar organizer regions (loops of DNA containing genes encoding rRNA), rRNA, ribosomal proteins, ribosomal-binding proteins, small ribonucleprotein particles and RNA polymerase I. The nucleolus typically exhibits fibrillar centers, fibrillar components and granular components. The fibrillar centers are regions where transcription does not take place, whereas the fibrillar components are more dense regions where transcription is underway. The granular components are regions where the ribosomal precursor particles are undergoing maturation.

Various sequences (reviewed elsewhere [143]) with as few as seven amino acids [144] have been identified that can be used to direct a protein to the nucleolus. The nucleolus localization signals (NoLSs) are very similar to the arginine/lysine-rich NLS sequences, which is easy to conceive owing to the necessity to first be transported into the nucleus before targeting the nucleolus. However, there is a distinction between NLSs and NoLSs as demonstrated through deletion analysis of the NoLS in the ORF57 protein (among others), resulting in diminished nucleolar localization while retaining nuclear localization [145]. Through alignment of various proteins containing NoLSs, Weber *et al*. identified a common NoLS motif (R/K)(R/K)X(R/K) [146]. This highly conserved sequence was also noted by Horke *et al.* after finding it to be important for nucleolar localization in the human La protein [147]. Although there is still not a consensus NoLS and nucleolar targeting is not as well characterized as other signals, the addition of short NoLS sequences has been demonstrated to direct proteins to the nucleolus [144,148], validating their use in targeting the nucleolus.

Proteins without an identifiable NoLS have been shown to localize to the nucleolus via interactions with other molecules at the nucleolus. One such example is nucleolin, a protein that may localize to the nucleolus partially due to RNA binding. However, the RNA binding domains are not sufficient to cause nucleolar localization of chimeric proteins [149]. Additionally, NoLS-containing proteins, such as NPM [150] and NOM1 [151], have been shown to bind and cause nucleolar localization of proteins that do not contain a NoLS, further expanding the mechanisms for nucleolar localization. Emmott and Hiscox have recently proposed that nucleolar **hub proteins** play an essential role in nucleolar localization [145]. Thus, nucleolar targeting can be achieved through NoLSs, by being dragged by a NoLS-containing protein, or potentially by binding to a nucleolar hub protein.

There are relatively few drugs that target the nucleolus or nucleolar components specifically, but actinomycin D has been shown to induce nucleolar localization due to the inhibition of rDNA transcription [152,153]. Phosphorylation is a critical modification in the regulation of nucleolar proteins; indeed, kinase inhibitors have drastic effects on the nucleolus [154–156]. One such inhibitor, the casein kinase inhibitor 5,6-dichloro-1-ribo-furanosylbenzimidazole, causes the nucleolus to disassemble [157]. An example of a more specific interaction involving a potential nucleolar targeting therapeutic is the peptide consisting of amino acids 26–46 of the $p19^{ARF}$ protein [158]. This peptide, modified with additional arginine residues to increase cellular uptake, has been shown to bind Foxm1b and send it to the nucleolus where it is unable to function as a transcription factor. Foxm1b has been shown to have a key role in the development of hepatocellular carcinoma [159], and the nucleolar sequestration of Foxm1b presents a novel therapeutic intervention.

Mitochondria

Mitochondria contain a double membrane composed of phospholipid bilayers with proteins embedded in them. This results in two aqueous spaces; the matrix and the intermembrane space. The inner membrane contains the proteins involved in the respiratory chain complex, ATP synthase and protein import machinery. The human mitochondrial matrix encloses a small genome (mtDNA) coding for 13 hydrophobic proteins, 22 tRNAs and two rRNAs [160,161]. The 13 hydrophobic proteins encoded in the matrix are all involved in the electron transfer system. The matrix is also the site for the citric acid cycle, urea cycle and fatty acid oxidation. Therefore, mitochondrial dysfunction contributes to a range of human diseases including obesity, diabetes and cancer. Mitochondrial targeting is mainly performed to interrupt mitochondrial function including energy production, oxidative stress and the apoptotic pathway.

Mitochondrial function

Mitochondria maintain an acidic inner space and an electrochemical potential across the inner membrane, which are utilized by the electron transport system to transfer electrons to oxygen. The electron transport chain passes electrons from the reducing equivalents (flavin adenine dinucleotide and nicotinamide adenine dinucleotide) to oxygen. During this process, protons are pumped from the matrix to the intermembrane space via NADH dehydrogenase, cytochrome *c* reductase and cytochrome *c* oxidase. As a result, an electrochemical gradient is established, which activates ATP synthase to pump protons back in and activates ADP to ATP conversion as the energy source for the cell [162].

In the mitochondrial respiratory chain, oxygen is partially reduced to form reactive oxygen species (ROS), which activate proton leak uncoupling proteins that pump protons back into the matrix without forming ATP [163–165]. Superoxides are also converted to other ROS such as hydroxyl radicals, which contribute to a number of degenerative diseases [166–170]. The oxidative damage, which increases with aging, also affects lipids, proteins and DNA, leading to a decline in the efficiency of oxidative phosphorylation [167,171]. Mitochondria destroy the free radicals using antioxidants (e.g., vitamin E, ascorbate and ubiquinol), which scavenge ROS converting them to less reactive species or prevent oxidative damage. Therefore, antioxidants have been used as supplements to specifically target the mitochondria (reviewed elsewhere [172]).

Apoptosis is frequently triggered through the mitochondria after collapse of the inner transmembrane potential, disruption of electron transport and ATP production, oxidative stress, permeability transition pore opening or mitochondrial swelling with outer membrane rupture. When the outer mitochondrial membrane undergoes permeabilization, cytochrome *c* is released from the intermembranous space into the cytosol activating caspase-3 via the Apaf-1 pathway [173–175]. Mitochondrial outer membrane permeabilization is commonly disabled in cancer cells and hence its pharmacological induction constitutes a therapeutic goal [176]. Bcl-2 and **Bcl-X_L** are mitochondrial proteins that inhibit apoptosis by inhibiting Bax and Bak oligomerization [177–181].

Mitochondrial import machinery

Drugs targeting mitochondria are necessary to induce apoptosis in cancer cells as well as to protect cells from oxidative damage and to repair defects. The outer membrane of the mitochondria allows diffusion of small molecules through pores formed by the spanning β-barrel protein porin. Simple ions with localized charge such as Cl^+, Na^+ or Ca^{2+} require translocators or energy-dependent transporters to allow them to cross into the matrix (reviewed in [182]). However, ions with delocalized charge and cationic hydrophobic molecules such as

triphenylphosphonium (TPP) can diffuse across the mitochondrial membrane taking advantage of the hydrophobic nature of the membrane [183–185]. Conjugating antioxidants to TPP or a methyl derivative increases their accumulation within the mitochondria and selectively blocks mitochondrial oxidative damage (Table 3). TPP cation has been covalently attached to antioxidants such as ubiquinol (MitoQ®) [186–188] and α-tocopherol (MitoE®) [189]. The same concept has been utilized to target DNA to the mitochondria using dequalinium, a delocalized cationic lipid [190]. It crosses the plasma membrane by endocytosis, and then DNA is released upon interacting with the mitochondrial membrane [191].

Large molecules (e.g., proteins) exploit the mitochondrial protein import apparatus to cross the outer and inner membrane. The TOM complex (translocase of the outer membrane of mitochondria) is the molecular machine responsible for translocating proteins across the mitochondrial outer membrane. The TOM complex contains receptor subunits, Tom70 and Tom20, which recognize proteins destined for import [192,193]. Other TOM complex components, Tom40, Tom22, Tom7, Tom6 and Tom5, assist the transfer of the protein into the intermembrane space [194–196]. The TIM complex (translocase of the inner membrane of mitochondria) binds to proteins destined for localization to the inner membrane and the matrix through TIM22 and TIM23, respectively [194,197]. The TIM22 complex translocates proteins depending on the electrochemical potential across the inner membrane. However, the TIM23 complex drives the protein trafficking via a motor complex built around a mitochondrial heat shock cognate protein 70 [193]. Mitochondrial targeting signals (MTSs) are mainly N-terminal cleavable amino acids 15–40 residues in length, which are positively charged with a notable absence of negatively charged residues [198]. These MTSs forming amphipathic α-helices are thought to be important for their recognition by the translocation machineries in the mitochondrial outer (TOM complex) and inner (TIM complex) membranes [193,197,199–202]. Once the protein is translocated into the matrix, the targeting signal is proteolytically removed by mitochondria processing peptidase [203,204]. Nevertheless, a significant fraction of mitochondrial proteins, especially proteins of the outer membrane, the inner membrane space and the inner membrane, lack typical N-terminal sequences and are targeted to the mitochondria by means of internal, noncleavable signals known as ‘carrier sequence motifs’ [205,206]. These internal signals resemble the N-terminal sequence and are recognized by the TOM machinery [207–209]. The internal signals function in combination with a preceding hydrophobic region [210].

Attaching MTSs to essentially any protein, DNA or RNA enables mitochondrial targeting [211–215] (Figure 2C & D). MTS has also been fused to restriction enzymes such as Sma1 endonuclease to degrade mutant mtDNA in neuropathy, ataxia and retinitis pigmentosa (NARP) disease [216]. Targeting proteins such as p53 to the mitochondria induces apoptosis by complexing with Bcl-X_L proteins. The inactivation of Bcl-X_L induces conformation change and oligomerization of the BH1,2,3 effector protein Bak and Bax, which then forms a pore in the outer membrane facilitating the release of cytochrome *c* [217–219]. We are currently investigating the fusion of a strong NES to small proteins tagged with MTS to reduce nuclear localization and increase their availability in the cytoplasm to target the mitochondria [Mossalam M, Opper K, Lim CS, Unpublished Data]. The effect of the different subcellular signals in a protein and the outcome of having multiple signals is a largely unexplored area, and warrants attention. Table 3 lists examples of chemical (TPP) and biological (MTS) means to target therapeutics to the mitochondria.

Endoplasmic reticulum

The ER is an organelle found in all eukaryotic cells. Its membrane represents as much as half of the total membrane of an average animal cell. It is continous with the nuclear envelope and extends to the cell periphery intertwining with most cellular organelles, including the

mitochondria, peroxisome and the Golgi apparatus [220]. Regions of the ER that are coated with ribosomes are called rough ER, where proteins are imported into the ER in a cotranslational process. In cotranslational transport, one end of the protein is translocated into the ER while the rest of the protein is being assembled in the ribosome. Smooth ER is the region that lacks bound ribosomes. An essential function of the ER is the biosynthesis of proteins and lipids that are destined for intracellular organelles and the cell surface [221]. It also controls Ca^{2+} signaling via its Ca^{2+}-binding proteins and homeostasis [222].

ER targeting

The cotranslational targeting of proteins to the ER membrane (transmembrane proteins) and lumen (water-soluble proteins) is mediated by cytosolic ribonucleoproteins called signal recognition particles (SRPs) and their cognate membrane-associated receptors [223–225]. One end of the SRP binds to the ER signal sequence as they emerge from ribosomes, while the other end of the SRP blocks the translational elongation in the ribosome halting protein synthesis [226–229]. The signal sequence consists of 7–12 large hydrophobic residues that presumably form an α-helix [229–233]. The SRP–ribosome complex is then directed to the ER membrane by binding to the SRP receptor, which is an integral membrane protein complex embedded in the rough ER membrane [224,234–237]. This binding releases the ribosome complex to the integral membrane protein Sec61 translocation complex (translocon), allows the translational elongation to continue and recycles the SRP back to the cytosol [238,239].

The ribosomal exit tunnel is aligned to a water-filled pore in the translocon through which the protein is translocated and continues being translated. Subsequently, the signal sequence is cleaved via an ER signal peptidase and released into the membrane, where they get degraded by other proteases. The translocon also allows a translocating protein lateral access into the hydrophobic core of the membrane for both integration of membrane proteins into the bilayers (via either a stop-transfer sequence or a signal-anchored sequence) and the release of the cleaved signal peptide into the membrane (for reviews see [240,241]). The ER is not limited to cotranslational targeting of proteins; some post-translational proteins are imported into the ER in a SRP-independent mechanism, where they depend on the hydrolysis of ATP and the binding to *cis*-acting molecular chaperones such as heat shock cognate protein 70 [242–247]. The signal sequences in this category are no more than 70 amino acid residues in length [245,248].

The ER signal sequences have substrate-specific differences, which may have functional consequences [249,250]. The signal sequences may differ in their gating of the translocation complex [251,252], dependency on the translocon-associated proteins [253,254], translocation efficiency of proteins [255–257] or sensitivity to translocation inhibitors [258,259]. Table 4 lists examples of ER signal sequences fused to therapeutic proteins.

ER function

Nearly a third of the eukaryotic genome is processed through the ER [260]. The transferred transmembrane proteins either function in the ER or reside in the membrane of other organelles or the plasma membrane. Similarly, the water-soluble proteins are either secreted to the cytosol or transferred into the lumen of other organelles. Transmembrane proteins directed to the ER include plasma and organelle membrane receptors and channels that regulate metabolic pathways, regulate cell-to-cell communication and flow of ions and solutes, and mediate protein and lipid uptake from the surrounding cellular environment. In addition, secreted proteins and enzymes are required for cell communication with its surroundings and to support sorting, metabolic and catabolic activities [261]. Therefore, a wide range of diseases occur owing to mistakes in protein handling in the ER such as cystic fibrosis, liver failure,

Alzheimer's disease, Parkinson's disease and diabetes mellitus [262–270] (for a list of diseases see elsewhere [261]).

The Golgi apparatus

The Golgi apparatus is known typically for post-translational modifications and shipping macromolecules to the plasma membrane, lysosomes or outside of the cell in secretory vesicles. Thus, the Golgi apparatus ships molecules in the opposite direction to drug delivery. Major players in this process, such as mannose 6-phosphate receptors, are recycled back to the trans-Golgi network in a retrograde fashion [271–273], demonstrating the potential for use in therapeutic targeting of the Golgi apparatus. However, this recycling pathway involves late endosomes [271,274,275] with the associated low pH and degradative enzymes that therapeutic delivery systems may want to avoid. However, retrograde delivery to the Golgi apparatus directly from early endosomes or recycling endosomes has been characterized with the nontoxic targeting subunit of toxic proteins such as Shiga and cholera toxins [276,277]. The receptor for these toxins is a glycolipid (globotriaosyl ceramide) that uses a pH-independent pathway [277] to target the Golgi apparatus and may have therapeutic potential. While clathrin is critical for efficient transport of Shiga toxin [278], ricin has been shown to transport to the Golgi apparatus independent of clathrin and Rab9-GTPase [279] in a process regulated by cholesterol [280]. Other proteins have also been shown to shuttle between the plasma membrane and the Golgi apparatus. One such example is a protein commonly used as a marker for the trans-Golgi network named TGN38/41, a receptor for p61 protein complexes and GTP-binding proteins, important in the formation of exocytic vesicles [281]. Other examples are furin [282] and carboxypeptidase D [283].

The signaling sequences used by these proteins to target the Golgi apparatus are not well characterized, and no specific consensus sequence has been proposed. However, tyrosine-containing motifs such as that found in TGN38/41 (SDYQRL) is one signal responsible for Golgi apparatus localization. Attachment of this signal to the low-density lipoprotein receptor has resulted in localization to the Golgi apparatus [284]. It has been shown that furin has two specific signals that target the Golgi apparatus: the acidic sequence CPSDSEEDEG, which is sufficient to cause localization to the Golgi apparatus; and the tyrosine-containing sequence YKGL, which has been proposed to function as a retrieval signal through targeting of endosomes [285,286]. The localization of furin to the Golgi apparatus, and its shuttling to and from endosomes, is further dependent on phosphorylation by casein kinase II at serine residues in the acidic cluster motif, and dephosphorylation by protein phosphatase 2A [287,288], highlighting the role of post-translational modifications on localization and not just specific amino acid sequences. A similar mechanism for targeting the Golgi apparatus via a tyrosine motif and a phosphorylation site has been demonstrated for the varicella-zoster virus glycoprotein I [289]. While sequences targeting the Golgi apparatus have been studied and identified, little effort has been put forth to harness these signals for therapeutic delivery to the Golgi apparatus, leaving much to be explored.

Although the Golgi apparatus may not be the target of the therapeutic, delivery of therapeutics via the B subunit of Shiga toxin, which enters the cell through the retrograde pathway involving the Golgi apparatus, have been studied for vaccination, targeted killing of cancer cells and imaging of cancer cells. Although the mechanism has not been delineated entirely, the MHC class I pathway involves the retrograde pathway through the Golgi apparatus, and Shiga toxin subunit B (STB) or Shiga-like toxin subunit B (SLTB) thus has been conjugated to various antigens for tumor protection [290–294]. The receptor that binds ST/SLT is the globotriaosylceramide receptor found to be overexpressed on many tumor cells, allowing the retrograde pathway with ST/SLT or STB/SLTB to be used for tumor imaging [295] and targeted delivery of cytotoxic agents for colon [296], ovarian [297] and breast cancer [298],

as well as lymphoma [298,299], astrocytoma [300] and meningoma [301]. Finally, since the Golgi apparatus has been described to exhibit a mechanism for stress-induced initiation of apoptosis [302], this validates future attempts at targeting therapeutics to the Golgi as a mechanism for specific induction of apoptosis in malignant cells.

Peroxisomes

The peroxisomes are multifunctional, single-membrane enclosed, spherical vesicles distributed throughout the cytoplasm [303]. Their numbers vary in different cell types and under different stimuli. The term 'peroxisome' was coined due to the fact that hydrogen peroxide is formed and degraded in the organelle. Peroxisomes are responsible for a wide variety of biochemical and metabolic pathways, with implications for human health. Aging and several diseases are associated with dysfunction of the peroxisomes, which has made it an interesting pharmaceutical target.

Peroxisomal function

A major function of the peroxisome is the breakdown of a variety of fatty acids via α- and β-oxidation reactions. Similar to mitochondrial β-oxidation, fatty acids are broken down two carbons at a time in the form of acetyl-CoA. However, fatty acids containing a methyl group at the three-carbon position must be modified before entering the peroxisomal β-oxidation pathway [304]. Many of the metabolic pathways in the peroxisomes lead to hydrogen peroxide production, which is subsequently metabolized by the peroxidase catalase. Peroxisomes also play a role in the production of bile acids, docosahexanenoic acids and ether phospholipids.

Peroxisomal import machinery

The import machinery to the peroxisomal matrix requires:

- Cargo containing specific targeting signals
- Receptors that recognize specific signals
- Membrane-associated import via docking and translocating proteins
- Cargo release (reviewed elsewhere [305])

Cytosolic proteins require specific targeting signals recognized by receptors for peroxisomal targeting. Two types of peroxisomal targeting signals exist: type I (PTS1) and type 2 (PTS2). PTS1 is the most abundant, which consists of a tripeptide (SKL) or a conserved biochemical variant (consensus S/A/C-K/R/H-L/M) at the extreme C-terminus of the protein [306–308]. An additional adjacent upstream region to the PTS also influences the interaction between receptor and PTS [308–310]. By contrast, PTS2 consists of a less conserved consensus sequence (R/K-L/I/V-X_5-Q/H-L/A, where X may be any amino acid) and is coded close to the N-terminus [311–313]. For a list of *bona fide* peroxisomal proteins and their PTSs, see [314].

Specific proteins called receptor peroxins (Pex5p and Pex7p) bind to the proteins harboring PTS in the cytosol, which then target the peroxisomal membrane as a receptor–cargo complex. PTS1 is recognized by Pex5p while Pex7p recognizes PTS2 [311,315–317]. Pex5p is retained in the cytosol in the tetramer form. Upon binding to proteins containing PTS1, Pex5p disaggregates into dimers [318]. Each dimer carrying two cargo proteins is then transported to the peroxisome. Subsequently, the receptor–cargo complex binds to membrane-associated peroxins (docking proteins), in particular Pex8p, Pex13p, Pex14p and Pex17p [319]. The remaining membrane-associated peroxins (Pex2p, Pex10p and Pex12p) form the RING–finger subcomplex. Both the docking proteins and the RING–finger complex form the putative import complex (importomer), which is thought to support translocation [320]. The precise mechanism for the peroxisomal protein import machinery remains ambiguous. The uniqueness in this

mechanism is highlighted in its ability to transport folded, cofactor-bound and oligomeric proteins [321]. There are two proposed models on the receptor's cycle between the cytosol and the peroxisome. The model of shuttling receptors states that the cargo dissociates from the receptor upon binding to the docking proteins [311,322]. The cargo is then translocated across the peroxisomal membrane while the receptor is released back to the cytosol. Alternatively, the extended shuttle hypothesis assumes that the dissociation between the cargo and receptor takes place in the peroxisomal matrix allowing the unloaded receptors to transport back to the cytosol [323–325].

Targeting the peroxisomal membrane (without translocating to the matrix) follows a different import mechanism. Peroxisomal membrane proteins (PMPs) require membrane protein targeting signals. These signals contain a cluster of basic amino acids in conjunction with one or more transmembrane regions downstream from it [326–329]. The membrane protein targeting signal is recognized by the Pex19p receptor in the cytosol, which then binds to Pex3p on the peroxisomal membrane. However, the exact mechanism on how PMPs are transported to the peroxisomal membrane is still unknown. There is also a possible but controversial involvement of the ER in PMP trafficking [305,330–332]. The importomer plays an important role for matrix targeting but not for membrane protein import.

Peroxisomal disorders are due to either mutations in peroxins (peroxisomal biogenesis disorder [PBD]) or deficiencies in peroxisomal enzymes (reviewed in [333]). PBDs are categorized by means of clinical severity into the Zellweger spectrum of disease (Zellweger syndrome, neonatal adrenoleukodystrophy) and infantile Refsum's disease or rhizomelic chondrodysplasia punctata (RCDP) type I. For example, a defect in the Pex7p disrupts enzymatic pathways for enzymes containing PTS2 causing RCDP type I. In addition to peroxin mutations that affect enzymes delivery to the peroxisome, single peroxisomal enzyme deficiencies have been linked to a number of human diseases such as adult Refsum's disease, X-linked adrenoleukodystrophy, RCDP type II and III, primary hyperoxaluria type I and acatalasemia (for a full list of diseases, see elsewhere [333]).

Peroxisomal targeting

Specific targeting to the peroxisome is of great clinical value to restore, alter or complement peroxisomal function. It could be used in the treatment of single enzyme deficiencies, PBD, as well as diseases associated with altered ROS [333]. Tagging protein therapeutics or peroxisomal enzymes to a PTS is required to achieve peroxisomal targeting. The carboxy-terminal PTS1 (SKL or related sequence mentioned previously) is better characterized and is easily tagged onto a protein of interest using standard recombinant molecular cloning techniques. The adjacent upstream region influences the receptor/PTS interaction and can be evaluated using a computer program [401], which can also predict the relative strength of a PTS1. To be recognized by Pex5p, accessibility of the PTS1 (not buried in the folded protein) is also crucial. Therefore, a spacer between the PTS1 on the carboxy terminal and the protein could be introduced. Table 5 lists therapeutic proteins delivered to the peroxisome via PTS1.

Proteasomal function, inhibition & potential drug delivery

The proteasome is an ATP-dependent prote-ase that functions to remove (degrade) abnormal proteins found in the cytosol, nucleus or ER. The entire proteasome (26S proteasome) consists of the 20S proteasomal core, capped by two 19S regulatory subunits on each end. Polyubiquitination of proteins triggers degradation by the 26S proteasome complex [19]. The proteasome has become a popular target for drug therapy, since inhibiting the proteasome can be used for treatment of inflammatory diseases and cancer [334]; on the other hand, activation of the proteasome has therapeutic use in neurodegenerative diseases and cancer [335].

Synchronized synthesis and degradation of regulatory proteins is necessary for cells to function and progress through the cell cycle. In the case of tumor development, if the degradation of these proteins can be blocked, apoptosis will ensue. Rapidly dividing cancer cells are more susceptible to proteasomal inhibition since they have more disruptions in their normal regulatory pathways than noncancerous cells [336]. Bortezomib is the first small-molecule inhibitor of the ubiquitin-protease system to be approved for treatment of multiple myeloma [337,338]. While bortezomib targets the 20S subunit of the proteasome, many other drugs are being developed to target other components of the proteasome [339,340] and other components (ubiquitin ligases, ubiquitin-activating and -conjugating enzymes) of the proteasomal degradation pathway [341]. In addition, aberrant proteins could be sent to the proteasome for degradation. One focus of our laboratory is a gene therapy approach to capture oncogenic proteins and send them to the proteasome for degradation.

Conclusion & future perspective

Protein subcellular localization is behind a wide range of diseases including cancer (for a list of diseases and proteins that require specific intracellular targeting, see [5]). While unidirectional targeting of a therapeutic agent to single organelles has been well studied and utilized, multidirectional drug targeting, utilizing multiple signals, represents a more sophisticated level of targeting that mimics the complexity of nature. In nature, many signal-transducing proteins are not simply directed to one compartment of a cell. They may interact with other proteins in multiple compartments of a cell. For example, the p14/19 ARF protein can bind Mdm2 (a nucleocytoplasmic shuttling protein) to inhibit degradation of p53 by Mdm2. The p14/19 ARF protein also contains a nucleolar signal, which can re-direct the p14/19 ARF–Mdm2 complex to the nucleolus [342–344].

While we have already demonstrated that unidirectional targeting of an oncogene can dramatically alter its function [4], our ultimate goal is to utilize our protein switch technology to capture an endogenous protein in one compartment, and move it to another compartment [6,7]. Our laboratory is pursuing this approach for treatment of CML. Bcr-Abl, the causative agent of CML, is found in the cytoplasm of CML cells, where it sends oncogenic signals to other proteins in the cytoplasm. If endogenous Bcr-Abl can be captured and dragged to the nucleus of cells, apoptosis will ensue owing to loss of oncogenic signaling in the cytoplasm and gain of apoptotic function in the nucleus. Like nature, the protein switch was designed to regulate cellular proteins by changing their location and hence their function. The protein switch has the advantage of being regulated by an externally added ligand. The protein switch contains a dimerization domain (to capture the endogenous protein of interest), a NES (conferring cytoplasmic localization) and a ligand-regulated nuclear import signal. Upon addition of the ligand, the protein switch is designed to translocate from the cytoplasm to the nucleus and drag its dimerization partner with it.

Other groundbreaking studies have included the concept of delivering drugs to multiple compartments (across multiple barriers) by mimicking viral delivery. A review by Wagner discusses mimicking viral delivery of DNA to the cytoplasm, and into the nucleus [345]. There are even virus-mimetic nanogels that can deliver drugs to not only targeted cells, but also to neighboring cells [346].

The future of drug delivery to organelles lies in our ability to more closely mimic, or even improve on, nature. Spatial placement of signaling sequences in a protein, balancing the strengths of different signals against each other and the masking/unmasking of signals due to conformational changes in an engineered protein or therapeutic have not been fully explored or understood. The ability to harness genetic codes, signal sequences, targeting motifs or protein–protein interactions will lead to enormous complexity and diversity for drug delivery.

Executive summary

- For maximum therapeutic effect and minimal side effects, drugs need to be delivered to the appropriate location within the cell.
- The cytoplasm and nucleus are the most widely targeted and well-studied organelles.
- Cytosolic targeting is crucial before most drugs can reach any organelle within the cell.
- Development of drug carriers to overcome barriers for cytosolic delivery and endosomal escape is still in progress.
- Localization and export of proteins to and from the nucleus can also be blocked using molecules that inhibit either the import or export mechanism.
- Gene delivery mainly targets the nucleus, while a few studies are targeting DNA to the mitochondria.
- Targeting specific organelles is under investigation, especially the mitochondria owing to its role in oxidative stress and apoptosis.
- Manipulating current therapeutics with specific targeting signals ensures accumulation in certain compartments within the cell.
- Capturing and sending oncogenic proteins to the proteasome is a promising approach for cancer therapy.
- Proteins synthesized at the endoplasmic reticulum are destined for intracellular organelles and the cell membrane.
- Newer technologies (e.g., protein switch and virus-like delivery systems) mimic nature and allow for a more sophisticated approach to drug delivery. Harnessing different methods of targeting multiple organelles in a cell will lead to better drug delivery and improvements in disease therapy.

Key Terms

Signal hypothesis	Proteins destined for movement across biological membranes contain amino acid sequences that may/may not be present in the mature protein
Stealth liposomes	Liposomes designed with a surface coating, intended for prevention of liposome binding with serum components (hence long circulation)
Proton sponge effect	Polymers (e.g., polyethyleneimine) with buffering capacities between 5 and 7.2 can potentially rupture the endosome
Nups	Abbreviation for nucleoporins, a family of proteins that compose the main component of the nuclear pore complex
Protein aptamer	Engineered protein that is soluble and stable inside of cells and binds to another macromolecule. It is composed of a peptide binding sequence attached at both ends to a scaffolding protein such as thioredoxin-A
Hub protein	A protein that interacts with a large number of other proteins

Bcl-X_L proteins	B-cell lymphoma-extra large' is a member of the Bcl-2 family of proteins. It is an anti-apoptotic protein found in the transmembrane of the mitochondria

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Website

401. PTS1 Predictor. http://mendel.imp.ac.at/pts1/

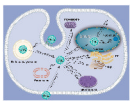

Figure 1. Drug targeting to cellular organelles

Text on arrows indicate consensus targeting sequence (if available), example of targeting signal or mode of targeting.

ER: Endoplasmic reticulum.

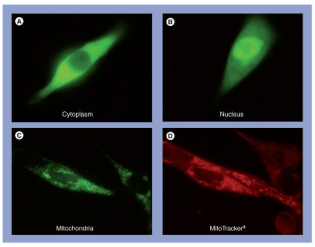

Figure 2. Enhanced green fluorescence protein fused to different targeting signals and transfected into 1471.1 murine adenocarcinoma cells

(A) Nuclear export signal (HIV NES) delivers enhanced green fluorescence protein (EGFP) to cytoplasm. **(B)** Nuclear localization signal (MycA8 NLS) delivers EGFP to nucleus. **(C)** Mitochondrial targeting signal (orthinine transcarbamylase MTS) delivers EGFP to mitochondria. **(D)** MitoTracker® (Invitrogen) stains mitochondria in live cells.

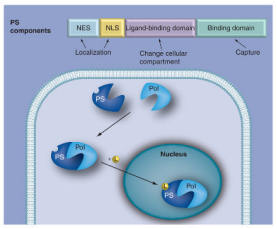

Figure 3. Protein switch mechanism

The PS, shown schematically (top diagram), consists of a NES, NLS, ligand-binding domain and dimerization domain. When PS plasmid is transfected into cells, the expressed PS captures a PoI in the cytoplasm. Upon addition of ligand, the PS–PoI complex translocates to the nucleus. L: Ligand; NES: Nuclear export signal; NLS: Nuclear localization signal; Pol: Protein of interest; PS: Protein switch.

Table 1

Cytosolic targeting of drug therapeutics (includes endosomal/lysosomal escape, in some cases).

Class of targeting agent	Targeting agent composition	Drug delivered (trade name if available)	Ref.
Liposomes	Liposome composed of lipid bilayer neutral lipids, DPPC and cholesterol	Amikacin (Arikace™)	[347]
	Liposomal includes soybean oil and phospholipids	Cyclosporin	[348]
	Doxorubicin STEALTH® liposome containing MPEG–DSPE, HSPC and cholesterol	Doxorubicin (Doxil®)	[349]
pH-sensitive liposomes	Liposomes composed of CHEMS and DOPE	Diptheria toxin A chain	[38]
	DOPE, *N*-succinyl–DOPE and PEG–ceramide	Gentamycin	[350]
Thermosensitive liposomes	DPPC, HSPC and cholesterol liposomes surface modified with DSPE-PEG-2000:PNIPAM-AAM17	Doxorubicin	[351]
Targeted thermal magnetic liposomes	DPPC, cholesterol, DSPE–PEG(2000) and DSPE–PEG(2000)–folate	Doxorubicin	[352,353]
Polymeric micelles	PEG–poly(aspartic acid) block copolymer	Doxorubicin (Adriamycin®)	[354]
pH-sensitive micelles	PEG-block-poly(aspartate-hydrazide) or PEG–p(Asp-Hyd) was modified using either levulinic acid or 4-acetyl benzoic acid attached via hydrazone bonds	Doxorubicin (Adriamycin)	[355]
pH-sensitive micelles with cell surface targeting	Amphiphilic block copolymers that self-assemble into spherical micelles, folate–PEG–poly(aspartate-hydrazone-adriamycin) with γ-carboxylic acid-activated folate	Doxorubicin (Adriamycin)	[356]
Thermosensitive micelles/polymers	Micellar cyclotriphosphazenes	Human growth hormone	[43]
	Biodegradable triblock copolymer of PLGA–PEG–PLGA (ReGel®)	Paclitaxel	[357]
Cell-penetrating peptides	Doxorubicin bound to HPMA-based polymer with the cell-penetrating peptide Tat	Doxorubicin	[358]
Cationic polymers and cationic lipids	Polyethyleneimine	Genes (DNA)	[61,359]
Virus mimetic	Hydrophobic polymer core (poly(L-histidine-co-phenylalanine)(poly(His_{32}-co-Phe_6) and two layers of hydrophilic shell (one PEG end linked to core polymer; other end to BSA)	Doxorubicin	[346]
NES	NES (LQLPPLERLTL) encoded in a plasmid	Genes (DNA)	[6,7,60]
	NES (ALPPLERLTL) conjugated to DNA	Antisense oligonucleotide	[360]

BSA: Bovine serum albumin; CHEMS: Cholesterylhemisuccinate; DOPE: Dioleoylphosphatidylethanolamine; DPPC: Dipalmitoylphosphatidylcholine; DSPE: Distearoylphosphatidylethanolamine; HPMA: *N*-(2-hydroxypropyl)methacrylamide; HSPC: Fully hydrogenated soy phosphatidylcholine; MPEG: Methoxypolyethylene glycol; NES: Nuclear export signal; PEG: Polyethylene glycol; PLGA: Poly(lactide-co-glycolide).

Table 2

Methods to link nuclear localization signals to DNA-based therapeutic agents.

Type of association	Description	Ref.
Electrostatic	NLS in complex with DNA	[361–365]
Electrostatic	NLS–PLy in complex with DNA	[366]
Electrostatic	M9–ScT in complex with DNA	[367]
Electrostatic	NLS–PNA in complex with DNA	[134,368–371]
Electrostatic	Triplex formation between padlock oligonucleotide with NLS and DNA	[372]
Electrostatic	NLS–streptavidin bound to biotinylated DNA	[373]
Covalent (random)	N3–adenine adduct via CPI–NLS	[120]
Covalent (random)	Photoactivation of p-azido-tetrafluoro-benzyl–NLS	[121]
Covalent (random)	Diazo coupling to increasing lengths of PEG–NLS	[122]
Covalent (specific)	Crosslinked triple helix with psoralen-oligonucleotide	[124]
Covalent (specific)	Attachment to hairpin of linear DNA	[125,126,128]
Covalent (specific)	Attachment to PCR primer	[126,127]

CPI: Cyclopropapyrroloindole; NLS: Nuclear localization signal; PEG: Polyethylene glycol; PLy: Polylysine; PNA: Peptide nucleic acid; ScT: SV40 T-antigen consensus NLS.

Table 3

Mitochondria targeting signals/motifs and delivered therapeutic agents.

Targeting motif	Therapeutic	Ref.
(2-oxo-ethyl)-triphenyl-phosphonium	Nitroxide (4-amino-2,2,6,6-tetramethyl-piperidine-1-oxyl)	[374]
Rhodamine β	Porphyrin	[375]
Stearyltriphenyl phosphonium	Liposome-based nanocarrier containing ceramide	[376]
Triphenylphosphonium	Ubiquinol antioxidant	[183]
Dequalinium	DNA	[190]
MLSCTSPLLRGACHNMGAAKALRLRWTVPPAVLIALG SGALYTTSGQTLYYKNSVQQTD	Phospholipase A ortholog (AoPlaA)	[377]
MLSRAVCGTSRQLAPALGYLGSRQ	Protein transduction domain of HIV-1 TAT protein	[378]
MSATRMQLLSPRNVRLLSRGRSELFAGGSGGGPRVRSLISP PLSSSSPGRALSSVSATRRGLPKEKMTENGVSSRAKVLT IDT	Alanine aminotransferase	[379]
MLFNLRILLNNAAFRNGHNFMVRNFRCGQPLQ	Tumor suppressor p53	[212]
MLFNLRILLNNAAFRNGHNFMVRNFRCGQPLQ	Dihydrofolate reductase	[213]

Table 4

Endoplasmic reticulum targeting signals/motifs and delivered therapeutic agents.

Targeting motif	Therapeutic	Ref.
MDSKGSSQKAGSRLLLLL VVSNLLLCQGVVSTP	Mammalian prion protein	[380]
ALAAALAAAAA	G-protein-coupled receptors	[381]
MRVLVLLACLAAASNA	Recombinant proteins in baculovirus expression vector system	[382]
MLLPVPLLLGLLGLAAAL and KDEL (ER retention)	Human papilloma virus-16 E7 antigen	[383]
MRYMILGLLALAAVCSAA	Vesicular somatic virus nucleocapsid protein and influenza virus nucleoprotein	[384,385]
MKFTVTFLLIICTLSAFC	Adenovirus type 5 E3 14.5-kD protein	[386]
METDTLLLWV LLLWVPGSTGD	TGF-β2 and -β3	[387]

ER: Endoplasmic reticulum.

Table 5

Peroxisomal targeting signals and therapeutics delivered.

Targeting motif	Therapeutic	Ref.
SKL	Catalase	[388,389]
SKL	Monohydroascorbate reductase	[390]
SKL	Serine:pyruvate aminotransferase	[391]
AKL vs NKF	Catalase	[392]