Subcellular targeting strategies for drug design and delivery

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Abstract | Many drug targets are localized to particular subcellular compartments, yet current drug design strategies are focused on bioavailability and tissue targeting and rarely address drug delivery to specific intracellular compartments. Insights into how the cell traffics its constituents to these different cellular locations could improve drug design. In this Review, we explore the fundamentals of membrane trafficking and subcellular organization, as well as strategies used by pathogens to appropriate these mechanisms and the implications for drug design and delivery.

The development of immunotoxins and lectin conjugates as 'magic bullets' that would direct a drug to specific target tumour cells bore enormous promise in the 1970s but did not bring about the change that was hoped for. Targeted drug delivery remains a considerable challenge[1,2]. Currently, the pharmaceutical industry relies on general molecular parameters such as the molecule's size, its ability to partition into hydrophobic solvents and its capacity to participate in hydrogen bonding[3] to ensure bulk delivery to the systemic circulation, ideally through the oral route[4]. This delivery is typically achieved at the expense of specificity in targeting the drug to the subcellular site of action.

Upon reaching the systemic circulation and subsequently the target organ or tissue, the drug binds to its target molecule, provided the target is localized at the plasma membrane. However, if the target is localized in intracellular compartments, the drug–target interaction could be impeded owing to the intracellular sequestration of the target. In this context, the bioavailable drug at the tissue of interest might not be able to inhibit or modulate its target. Currently, drug delivery to subcellular compartments is achieved by designing or identifying membrane-permeant drugs, which diffuse through intestinal and target cell membranes to pervade the entire cell. However, the diffuse presence of the drug might lead to non-specific interactions. These issues of subcellular availability and accessibility of a target molecule to a drug are of crucial importance in drug delivery, and alternative approaches are now being pursued to address this issue[1] (TABLE 1).

Cells constantly renew their constituents and traffic them to their respective locations. Therefore, understanding the cellular machinery itself could shed light on how specific drug targeting can be achieved. In the case of eukaryotic cells, newly synthesized proteins destined for various intracellular organelles contain sorting signals. These molecular 'zip codes' are recognized by the sorting machinery that targets the proteins to their respective compartments[5]. Similar signals participate in retrieving proteins from the plasma membrane and sending them to intracellular compartments, for either degradation or recycling, through endocytosis. The sorting machineries involved in biosynthetic and endocytic trafficking use a range of adaptors, retrieval proteins, coat proteins, Rab GTPases and soluble *N*-ethylmaleimide-sensitive factor accessory protein receptor (SNARE) proteins to ensure precise targeting to distinct organelles. Viruses and toxins, the host targets of which are localized in specific intracellular compartments, use host trafficking machinery to gain access to these targets[6].

Even at the plasma membrane, proteins and lipids are differentially sorted to different domains of the plasma membrane. For example, lipid rafts, which are dynamic cholesterol–sphingolipid assemblies, have key roles in signalling and pathogenesis[7] (BOX 1). Proteins also show preferential partitioning into these domains, and so raftophilic molecules are potentially of interest for targeting drugs to lipid-raft-preferring proteins. Other membrane domains such as Rab domains[8] or ceramide domains[9] that are present in intracellular compartments play crucial parts in various disease processes and are also of interest for targeted inhibition. Here, we review the sorting mechanisms that are essential for drug targeting to these compartments and consider potential trafficking-based targeting strategies that determine drug activity at specific subcellular sites. Like a letter sent in the post, drugs or molecules (the 'message') could be targeted by using specific sorting moieties (the 'address') to deliver the drug to the appropriate cellular location.

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doi:10.1038/nrd2897

Table 1 | **Drugs that are directed to subcellular targets and their applications**

Subcellular target	Drug or compound	Mode of action	Applications
Plasma membrane	Enfuvirtide C34	Lipid linkage promotes membrane targeting of inhibitors and better inhibition of the HIV fusion complex	HIV fusion inhibitors
	Myr-proS1	Myristoylated or stearoylated ProS1 domain of HBV targets the fusion complex	HBV fusion inhibitor
	Pepducin	Palmitoylation of the i3 loop of GPCRs efficiently modulates GPCR signalling	GPCR modulator
Early endosomes	Cholesterol-linked β-secretase inhibitor	Addition of cholesterol promotes membrane tethering and endocytosis into endosomes, in which the active enzyme is localized	Efficient inhibition of the β-secretase enzyme; a therapeutic target in Alzheimer's disease
	Anticancer drugs,	Ligands of cell surface receptors (folate, LDL cholesterol and transferrin) mediate endocytosis of the heterologous conjugates	Effective transport of anticancer drugs to the interior of the cell
	Denileukin diftitox IL-4 and IL-13	Diptheria toxin conjugates and Pseudomonas exotoxin conjugates enable the uptake of the IL and release in the intracellular site	Malignant lymphomas
Late endosomes and lysosomes	β-glucosidase, β-hexosaminidase	Replacement of lysosomal enzymes directly or by mannose-6-phosphate-mediated uptake	Enzyme replacement therapy for lysosomal storage diseases (Gaucher's and Fabry's disease)
	Cyclo-2	Cholesterol-sequestering agent	Niemann–Pick's disease
Endoplasmic reticulum and Golgi complex	Antigenic peptides	Delivery of conjugated antigens for presentation on MHC class I complex by conjugation to STX-B	Malignant lymphomas, ovarian cancer and intestinal adenocarcinomas
	Fluorescent cancer imaging dyes	Imaging of tumours by STX-B conjugates of fluorescent dyes, as STX-B binds to GB3, which is overexpressed by tumours	Colon cancer, liver metastasis and ovarian tumours
	Shiga holotoxin	Selective killing of GB3-overexpressing tumours	
Cytosolic delivery	Anticancer drugs, siRNAs and plasmids	Conjugation with cell-penetrating peptides such as Tat and VP22 enables transport of heterologous conjugates into the cell	Delivery of anticancer drugs, siRNAs, plasmid DNA and proteins
Mitochondria	Gamitrinibs	Selective targeting of the HSP90 network in cancerous mitochondria	Rapid tumour cell death
	Antioxidant (ubiquinol and α-tocopherol)	Lipophilic cations such as triphenylphosphonium cations conjugated with antioxidants target mitochondria and confer protection	Neurodegenerative diseases
Nucleus	Antitumour drugs, cisplatin, doxorubicin and DNA	Delivery of genes by viral-mediated vectors, viral-like particles or liposomes Nanoparticles encapsulate the drug and enable slow and effective release Targeted charge-reversal nanoparticles carry conjugates to the nucleus	Carcinomas

GB3, globotriaosylceramide (also known as CD77); GPCR, G protein-coupled receptor; HBV, hepatitis B virus; HSP90, heat shock protein 90; IL, interleukin; LDL, low-density lipoprotein; MHC, major histocompatibility complex; siRNA, small interfering RNA; STX-B, Shiga toxin subunit B; Tat, transactivator of transcription protein; VP22, viral protein 22.

Targeting to the plasma membrane

The plasma membrane is an important site for cellular signalling events, and many proteins of therapeutic interest are localized in this compartment[10,11]. Targeting drugs to these membrane-embedded proteins does not require substantial modification, as extracellular availability alone should facilitate drug–target interaction. However, there is considerable evidence that the efficiency of these drugs could be enhanced by modifications that increase their affinity for the plasma membrane. For example, the membrane affinity of peptide hormones determines their biological activity[12,13]. Membrane anchoring, through either lipid or protein conjugation, increases the concentration of the drug at the target membrane and confines the drug to subdomains therein, thereby increasing the effective concentration at the membrane and decreasing the half-maximal inhibitory concentration (IC_{50}) of the compound. Membrane anchoring also reduces the dimensionality of a drug, increases the half-life of the compound and/or enables efficient inhibition of conformation-specific events at the membrane[13–15]. The reaction rates of interaction between two membrane-anchored molecules are enhanced if the anchored molecules are confined to a subregion of the membrane (such as a lipid raft domain) at which the target is localized. This limits the diffusion of the molecule in the confined area and thereby would also increase the diffusion-limited interaction[15]. We briefly describe some examples in which membrane anchoring of inhibitors to plasma membrane proteins was successfully achieved.

Targeting HIV by membrane-anchored inhibitors. HIV-1 hijacks raft domains at the plasma membrane to enter T cells. The envelope protein of HIV-1 is composed of two proteins, glycoprotein 120 (gp120) and gp41, which are essential for virus fusion with either the host cell plasma membrane or endosomes. Enfuvirtide (Fuzeon/T-20; Roche/Trimeris), a 36-mer synthetic peptide fusion inhibitor derived from the gp41 region overlapping with its carboxy-terminal heptad region[16,17], inhibited the conformational change that is essential for HIV fusion and thereby inhibited HIV entry *in vivo*. However, large amounts of the inhibitor are needed to bring about inhibition. Membrane anchoring of enfuvirtide peptides by conjugation to a transmembrane domain of the human low-affinity nerve growth factor inhibited HIV infection with 100-fold higher efficiency than the soluble counterpart[18]. Apart from the reduction in dimensionality of the peptide, membrane-anchored enfuvirtide interacted preferentially with the pre-fusion conformation of gp41, further increasing efficiency[19]. This example provides a proof of principle for increasing drug efficacy by adding a membrane-targeting tag an inhibitor of virus entry.

Membrane anchoring of a different HIV fusion inhibitor with a lipid anchor (C34-Chol) also demonstrated enhanced potency[20]. Cholesterol anchoring facilitated specific targeting to raft domains, where HIV fusion occurred, demonstrating the therapeutic power of reducing the compound's dimensionality by membrane anchoring and raft targeting. Although HIV fusion is presumed to occur at the plasma membrane, a new study using high-resolution time lapse imaging suggests that the fusion of HIV occurs in the endosomes[21]. Lipid-linked inhibitors can act at the plasma membrane but they can also be endocytosed and thus inhibit endosome-associated processes[22,23].

Inhibition of hepatitis B virus (HBV) entry. Similar to inhibition of HIV entry, lipidated membrane-anchored peptides derived from surface glycoproteins exhibited inhibitory effects against HBV infection[24]. The envelope of HBV consists of large, middle and small proteins. Peptides derived from the proS1 domain of these proteins, when myristoylated or stearoylated, inhibited HBV entry efficiently. Acylation also increased their membrane partitioning and intracellular staining[25]. When the acylation of the peptides was modified to stearoylation, the inhibitory activity improved dramatically and was observed at picomolar concentrations. Lipidated preS1 peptides efficiently inhibited HBV infection *in vivo*.

Modulation of G protein-coupled receptor (GPCR) signalling. Owing to their involvement in many disease processes, GPCRs are important drug targets[26]. Ligand-induced conformational changes of transmembrane domain 3 (TM3) and TM6 of GPCRs initiate downstream signalling, and the third intracellular loop (i3 loop) is essential for the coupling between the receptor and the G protein[27]. Soluble peptides corresponding to the loop regions have been shown *in vitro* to modulate G protein activation but failed to act *in vivo*, perhaps owing to their inability to access the cytosolic side of the membrane-associated GPCR. Attaching a palmitate group to the i3 peptide sequence enabled the modified peptide (termed pepducin) to cross lipid bilayers and efficiently inhibit receptor activation[28]. The non-palmitoylated peptide neither crossed the membrane nor inhibited GPCR signalling. Such pepducins have resulted in the production of potent prophylactics against thrombotic complications associated with stroke[29]. Although different GPCRs differ in function and protein sequences, their mechanism of activation is highly conserved. Therefore, targeting the i3 loop of any GPCR with the corresponding pepducins might be a general strategy for inhibiting GPCR signalling.

Intracellular membrane trafficking

Many drug targets are localized to intracellular compartments such as the cytosol, endosomes, lysosomes, the Golgi complex, the endoplasmic reticulum (ER), mitochondria and the nucleus[6,30,31]. In certain cases, the drug target is distributed in several cellular compartments but resides in an active conformation in only one compartment[32]. Targeting drugs to these locations is a challenge, but the normal cellular machinery is able to overcome such challenges by making use of sorting signals[33].

An increasing number of endocytic pathways are being defined (FIG. 1). This heterogeneity in endocytic mechanisms ensures that different cargoes are internalized to their specific locations and are subjected to different interactions during this process[34]. We briefly review these various routes to gain a better understanding of their potential for drug targeting.

Clathrin-mediated endocytosis. This endocytic route engages mainly receptor–ligand complexes[35] (FIG. 2). Upon binding of the nutrient or ligand to its cognate receptor, sorting motifs in the cytoplasmic tail of the transmembrane receptors engage adaptor proteins, such as adaptor protein 2, which interact with clathrin triskelions to initiate the formation of clathrin-coated pits[36,37]. The invaginated pits are released into the cytoplasm as vesicles, aided by a small GTPase called dynamin that facilitates the fission process[36]. After delivery to early (or sorting) endosomes, the endocytosed cargo is recycled, sorted for degradation or delivered to the Golgi complex (FIG. 2).

Following the release of ligands from internalized receptor–ligand complexes in early endosomes, proteins such as transferrin receptors are recycled to the plasma membrane by one of two distinct recycling routes. One route involves RAB4 in direct sorting of the cargo from the endosomes to the plasma membrane[38]. By contrast, RAB11 regulates the sorting of the cargo from early endosomes through the perinuclear recycling compartment (FIG. 2). Control of recycling versus degradation is crucial as deregulation of this process could lead to cancer[39]. Contrary to the previously accepted tenet that endocytosis downregulates signalling, recent work suggests that endosomes contain signalling-active molecules that ultimately lead to nuclear signalling[40–42] and encourages investigation into targeted inhibition in these endosomes[43] for therapeutic purposes. For example, effective signalling from

Endosome
A membrane-bound vesicle that is formed by the invagination of the plasma membrane during endocytosis.

Clathrin triskelion
A clathrin structure that consists of three heavy chains and three light chains that weave together to form three 'legs' radiating from a central point. The heavy chains form the backbone whereas the light chains are involved in the formation of clathrin lattices.

Box 1 | **Lipid rafts in pathogenesis**

Lipid rafts are dynamic nano-assemblies in cell membranes that are enriched in cholesterol and sphingolipids[7]. Physicochemical differences in membrane lipids produce lateral heterogeneity in the membrane. The formation of these domains and the partitioning of proteins into them are dynamic processes[144], which makes direct visualization of these domains by conventional microscopic means difficult. However, higher-order cross-linking of either the raft lipids or proteins leads to stable raft clustering and the formation of domains that are easier to visualize[7]. Such a clustering process modifies the properties of raft domains, leading to the budding of these domains. This raft-induced budding is often used by viruses and pathogens to gain entry into the cell by triggering clustering of raft components[145]. In some cases, budding aids the release of the virus from the cell[146].

Pathogens use lipid and protein components in the rafts as receptors for gaining entry into the cell[6]. The protective antigen of anthrax toxin binds to cellular glycophosphatidylinositol-anchored proteins to enter the cell, whereas cholera and Shiga-like toxins use gangliosides[6]. HIV uses CD4 and chemokine receptors as entry receptors, both of which are partitioned in lipid rafts[147]. Host cell rafts assist HIV entry and also the assembly and subsequent release of the virus[148]. This results in viral envelopes that are enriched in raft lipids[145]. Therapies are aimed at multiple targets to inhibit raft formation or assembly as a means to inhibit HIV infection[149]. Raft-targeting of HIV entry inhibitors by cholesterol modification of fusion inhibitors shows promise[20]. Ectopic application of raft-disrupting agents (disrafters) has been successfully used in preventing vaginal HIV infection in transgenic mice[150]. Disrafters could be used in microbicide gels[151]. Identifying disrafters that are specific for rafts characterized by particular proteins poses a challenge, but these findings encourage further exploration[152].

In the case of influenza, the virus uses raft lipids to release itself from the cell[146,153]. The envelope components associate with raft lipids after biosynthesis and become apically sorted in epithelial cells. Once these proteins reach the apical surface, the host raft lipids provide a platform for virus budding to occur. The processes that involve raft lipids are potential targets for raft-directed inhibition[146,153,154].

Parasites such as *Plasmodium* use host raft domains in the erythrocyte for their entry[155]. Therapies based on raft targeting are being investigated for infectious diseases caused by protozoans[156,157].

Amyloidogenic peptides also rely on raft domains for pathogenicity. In the case of the production of β-amyloid peptide, the amyloidogenic secretases cleave amyloid precursor protein (APP) in raft domains to release the peptide[63,158]. The conversion of the cellular prion protein to the scrapie version occurs in detergent-resistant domains[159]. Internalization of APP and cellular prion protein, which plays a crucial part in the production of the amyloidogenic peptides, has been shown to occur in a unique clathrin- and raft-dependent endocytic route. Additionally, both prions and β-amyloid peptides use raft domains for their oligomerization[159]. In the case of mast cells, signalling mediated by $F_C\varepsilon$ receptor 1 occurs in lipid rafts[160]. Upon binding the immunoglobulin E–antigen complex, the receptor undergoes oligomerization in rafts and mediates signalling from these domains. Raft partitioning of the receptor is crucial in regulating and integrating signal progression and for the subsequent degranulation[160]. Treatment with disrafting molecules, such as small-chain ceramides, inhibits mast cell degranulation[161].

Prodrug
A drug that is designed to release the active moiety only upon certain activating conditions.

Transition state inhibitor
Inhibitors that are designed to mimic the transition state of a substrate molecule in the enzyme–substrate catalytic reaction. Such inhibitors do not undergo catalysis and inhibit the enzyme at the substrate-binding site.

epidermal growth factor to the nucleus has been found to be mediated by a subset of APPL1 (adapter protein containing PH domain, PTB domain and leucine zipper motif 1)-positive early endosomes[44]. Similarly, sustained nuclear signalling by signal transducer and activator of transcription 3 requires the trafficking of MET from the plasma membrane to the perinuclear endosomal compartment[45]. SARA (Smad anchor for receptor activation) endosomes engaged in transforming growth factor-β signalling are crucial for morphogen gradient formation and asymmetrical cell division[46,47]. In the case of the δ-opioid receptors, signalling molecules such as β-adrenergic receptor kinase 1 (also known as GRK2) have been shown to associate with the receptor in endosomes only after endocytosis[48]. Targeted inhibition of these endosomes[43] could be an effective strategy for the treatment of cancer and related diseases.

Raft-mediated endocytic routes. Many proteins, lipids, viruses[49,50] and toxins are internalized by routes that are non-clathrin mediated[51,52]. Cholesterol plays a crucial part in these modes of internalization, whereas dynamin is essential for only some cargoes. Cholera toxin, viruses, bacterial toxins and plant toxins are internalized by this route to the ER. Internalization of interleukin-2 receptor subunit-β is regulated by dynamin, ras-related C3 botulinum toxin substrate 1 (RAC1), PAK1, PAK2 and RhoA GTPases[53,54]. By contrast, glycophosphatidylinositol (GPI)-anchored proteins are internalized by a raft-dependent pinocytic route termed the GEEC (GPI-anchored protein-enriched early endosomal compartment) pathway[55]. This route is strictly dependent on cell division cycle 42 (CDC42) but is dynamin-independent and bypasses the sorting to early endosomes by using long invaginations from the surface (in contrast to the small vesicular endocytic carriers of the caveolar and clathrin pathways)[56] (FIG. 1).

In some cases, there is no clear distinction between the clathrin- and raft-mediated internalization routes, and there seems to be some interplay between these two routes. Tetanus toxin and amyloid precursor protein require a cholesterol-dependent pre-clustering process before they can be internalized through the clathrin-dependent route[57,58] (FIG. 1). This heterogeneity in the endosomal system allows for specific targeting of drugs to various compartments.

Targeting to intracellular compartments

Targeting to early endosomes. Early endosomes, which have lower pH than the extracellular environment, serve as sorting stations for endocytosed proteins. The unique pH environment of the endosomes regulates the activity of endosome-specific enzymes and is used by pH-dependent pore-forming toxins to disrupt the endosomal membrane[59,60]. The low pH of the endosomal lumen also allows the design of pH-dependent prodrugs[61] against therapeutic targets in diseases such as cancer and Alzheimer's disease.

The importance of early endosomal targeting was demonstrated in the case of β-secretase[23], the rate-limiting enzyme in the formation of the neurotoxic amyloid-β peptide[62]. β-secretase contains a cytoplasmic sorting motif that directs the enzyme into early endosomes. Although present at the cell surface, the enzyme is not active until internalized into early endosomes, in which the endosomal pH (~6) is optimal for its activity[32]. Therefore, most transition state inhibitors against β-secretase that are active in cell-free assays but do not penetrate into the endosomal lumen fail to act in cells and *in vivo*. Moreover, β-secretase partitions into raft domains, which seems to be essential for its catalytic activity. Both endosomal localization and raft partitioning modulate enzymatic activity[63]. As a proof of principle, effective β-secretase inhibitors that exploit both of these properties have been constructed[32,63] (FIG. 3).

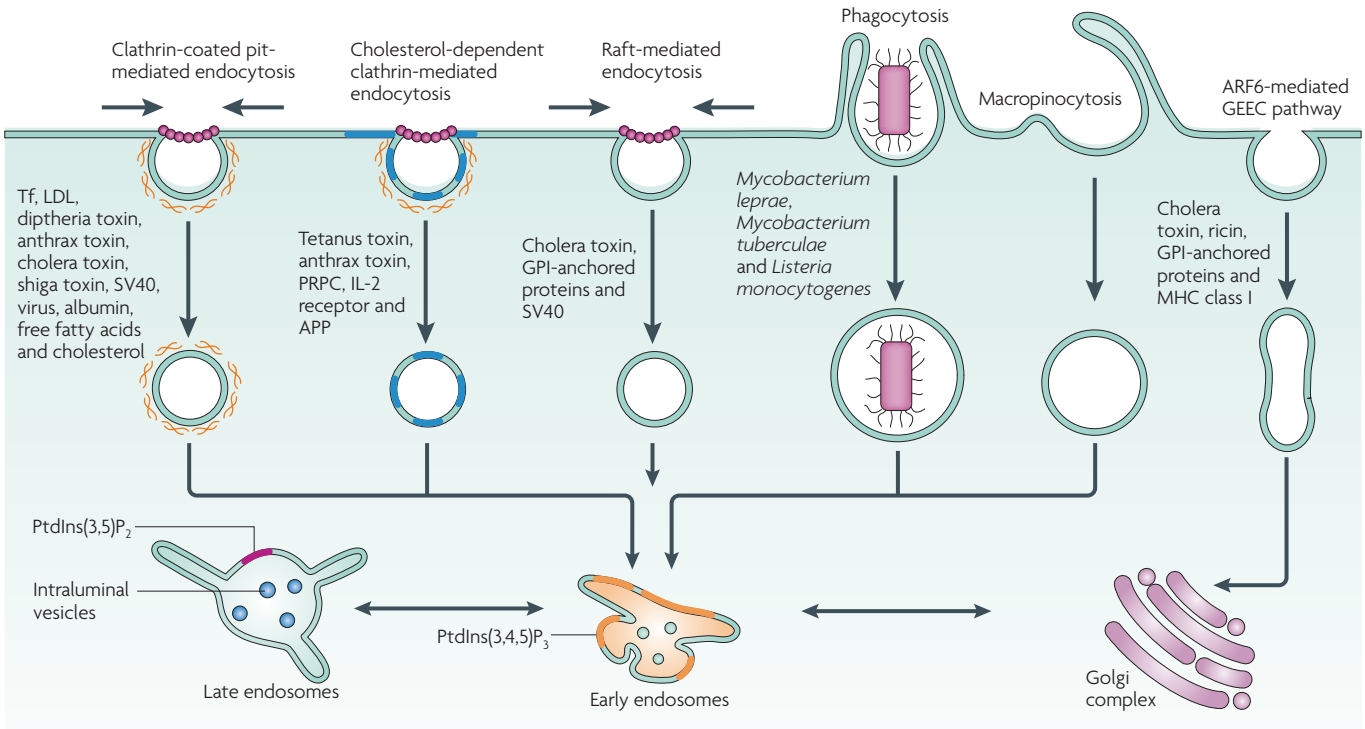


Figure 1 | **Heterogeneity of internalization pathways.** Cargoes such as transferrin (Tf) and low-density lipoprotein (LDL) are internalized through pits coated with clathrin (depicted as orange strips) that are pinched off into the cell employing the GTPase dynamin (shown as pink circles). Other cargoes such as anthrax toxin, cellular prion protein (PRPC), amyloid precursor protein (APP) and tetanus toxin are internalized through clathrin-coated pits but require cholesterol (shown as blue bars) for their endocytosis. Cholera toxin, glycophosphatidylinositol (GPI)-anchored proteins and some viruses are internalized through raft-mediated endocytic pathways. Larger particles, bacteria and viruses are internalized through phagocytic or macropinocytic pathways. Phagocytic internalization occurs with the stimulation of phagocytic receptors, which leads to the reorganization of membrane in an actin-dependent manner to form pseudopods. The pseudopods extend to engulf the particulate matter. Engulfed material in phagosomes undergoes fusion with lysosomes, a process that is inhibited by several intracellular pathogens. Cholera toxin and GPI-anchored proteins could also be internalized through ADP-ribosylation factor 6 (ARF6)-mediated GPI-anchored protein-enriched early endosomal compartment (GEEC) pathways that use long invaginations for transporting the cargoes into the cell. IL-2, interleukin-2; MHC, major histocompatibility complex; PtdIns(3,5)P_2, phosphatidylinositol-4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate; SV40, simian vacuolating virus 40.

Cholesterol linking led to membrane anchoring and enabled the inhibitor to be localized to raft domains, which are enriched for β-secretase. This modification reduced β-secretase activity more efficiently than its soluble counterpart *in vitro* and *in vivo*. The success of this strategy encourages further examination of lipid modifications to optimize drug delivery. However, these studies[20,23,25] were proof-of-principle studies, and several issues need to be addressed before proceeding to clinical studies. Lipid modifications could cause the drugs to be non-specifically adsorbed onto the membranes at the injection site and might therefore reduce the bioavailability of the compound. Cholesterol-modified drugs might be trafficked to the liver for detoxification and also affect the bioavailability of the drug. One other issue with targeted compounds is their stability. Future work should address these issues.

Several anticancer drugs have been targeted to endosomes by conjugating drugs to ligands that are internalized to endosomes through receptor-mediated interactions. Ligands such as transferrin[70,64,65], folate[66] and low-density lipoproteins[67] have been exploited for drug targeting, as these ligands show high affinity for their cognate receptors. As these receptors are often overexpressed in malignant tumours, conjugation of drugs or dyes to the ligands of these receptors aids specific tumour targeting for therapy or imaging[68]. However, the level of overexpression that is required to see differences between the tumour and normal tissue remains unclear.

Iron-loaded transferrin binds to the transferrin receptor (TFR) — a transmembrane protein that is present in almost all cells — and is sorted to early endosomes, in which the ligand is released from its receptor. Release enables the delivery of transferrin (or transferrin-conjugated drugs) to the early endosomal lumen and sorting of the receptor to recycling compartments. Antitumour drugs such as doxorubicin and cisplatin have been coupled to transferrin and showed more cytotoxic potency than unconjugated drugs[69,70].

Bioavailability
The extent to and rate at which the drug enters the systemic circulation.

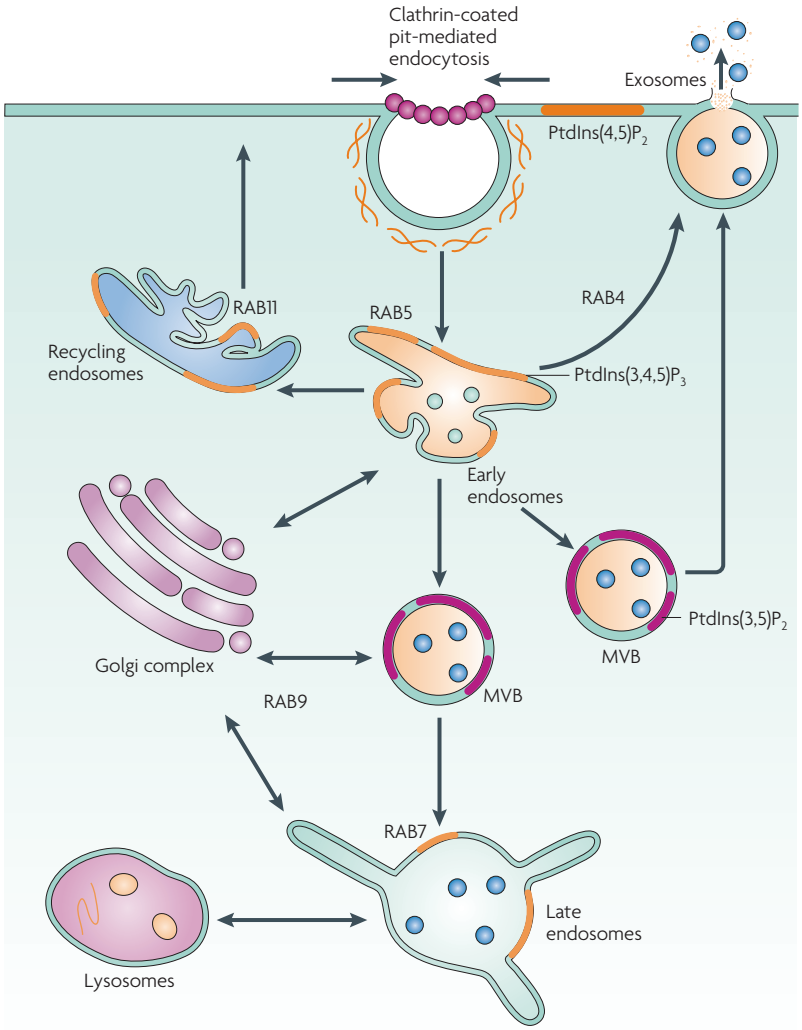


Figure 2 | **Clathrin-mediated endocytic pathway.** Proteins that are internalized through pathways mediated by clathrin (depicted as orange strips) and dynamin (shown as pink circles) are first sorted to early endosomes, which are characterized by the presence of the Rab GTPase RAB5. RAB5 participates in the fusion of early endosomes and the switch between RAB5 and RAB7 mediates the conversion of these endosomes to late endosomes. It is thought that, in many cases, an intermediate organelle called the multivesicular body (MVB; also known as the endosomal carrier vesicle) mediates the cargo transfer. Recent work has shown that ceramide is involved in the biogenesis of MVBs, which distinguishes these vesicles from late endosomes. Lipids and proteins sorted to the intraluminal vesicles (shown as blue circles) of MVBs can be released into the extracellular space by fusion of the MVBs with the plasma membrane as exosomes. Exosomes are implicated in various processes including antigen presentation, signalling and release of pathogenic peptides, such as prions and β-amyloid pepides. These pathogenic processes could be inhibited by specifically directing the inhibitors to the exosome-specific MVBs. Proteins destined for degradation, such as the epidermal growth factor receptors, are sorted from late endosomes to lysosomes, although the exact sorting mechanism is unclear. The low pH of lysosomes facilitates the activation of enzymes that are responsible for cargo degradation; this is a key consideration for drug design and delivery of pH-sensitive molecules. Proteins and lipids can also be directly recycled back from early endosomes to the plasma membrane by RAB4 GTPase. Recycling of some proteins to the cell surface occurs through recycling endosomes, marked by RAB11. Cargo transfer from endosomes to the Golgi complex is carried out by Rab proteins, such as RAB9 in the case of late endosomes. Intracellular organelles are also marked by the presence of unique phosphoinositide phosphates — namely, phosphatidylinositol-4,5-bisphosphate ($PtdIns(3,5)P_2$) and phosphatidylinositol-3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$) (shown as coloured arcs on the endosomes).

The folate receptor is also of interest for cancer therapy as it is upregulated in many epithelial cancers[68,71]. Folate receptors are GPI-anchored and internalized by a non-clathrin, non-dynamin, CDC42-dependent raft pathway[56]. Folate (and folate analogues such as petorate) have high receptor affinity (dissociation constant in the range 10^{-10} M), which enables efficient binding of folate conjugates to the receptors[66]. Folate receptor-targeted delivery of liposomal doxorubicin to folate receptor-expressing cells increased the cellular uptake and cytotoxicity of doxorubicin[72]. Polyethylene glycol–folate conjugates of thioctic acid carrying gold nanoparticles were used against ovarian cancer cells and were also faithfully targeted to the endosomal compartment[73]. Other ligands such as vitamin B12 and low density lipoprotein-like nanoparticles have also been used as drug conjugates[67]. In some cases, conjugates directed to TFR are shown to be not only targeted to endosomes but also imported into the brain. These results suggest that TFRs could be used to deliver drugs targeting the central nervous system[74–76]. Their broader use remains to be demonstrated.

Targeting to late endosomes and lysosomes. Lysosomes contain ~40 different hydrolytic enzymes that mediate controlled intracellular degradation of macromolecules. These organelles are of particular interest for the design and delivery of pH-dependent prodrugs and for devising strategies to inhibit degradation in these compartments.

Genetic deficiency in lysosomal hydrolases or proteins involved in the efflux of metabolites causes lysosomal storage diseases[77] in which accumulation of undigested metabolites often results in neurological symptoms. These diseases also arise owing to mutations that lead to defective localization or trafficking of lysosomal hydrolases to lysosomes from the ER or Golgi complex[31]. In many cases, enzyme replacement therapy remains the most successful and viable option for patients with lysosomal storage diseases[78,79]. These enzymes are normally targeted to lysosomes but in some cases the expression of certain surface receptors, such as the mannose-6-phosphate receptors[80], has been found to upregulate the delivery of the exogenous enzyme to macrophages in patients with Gaucher's disease. Dexamethasone, which is known to upregulate mannose-6-phosphate receptors, can be administered with the recombinant enzyme to enhance the uptake of the enzyme in macrophages[81].

Niemann–Pick disease type A and Niemann–Pick disease type B are caused by defective sphingomyelinase activity in lysosomes, whereas Niemann–Pick disease type C is caused by the loss of the polytopic transporter Niemann–Pick C1 protein that leads to accumulation of cholesterol in late endosomes and lysosomes[82]. Defective cholesterol trafficking has been implicated in Niemann–Pick's disease and a single injection of the cholesterol-sequestering agent 2-hydroxypropyl-β-cyclodextrin (also known as cyclo) ameliorated the severity of the disease in a mouse model[83].

Drug targeting to the ER and Golgi complex. Various clathrin-independent pathways traffic toxins such as Shiga or cholera toxins into the Golgi and ER

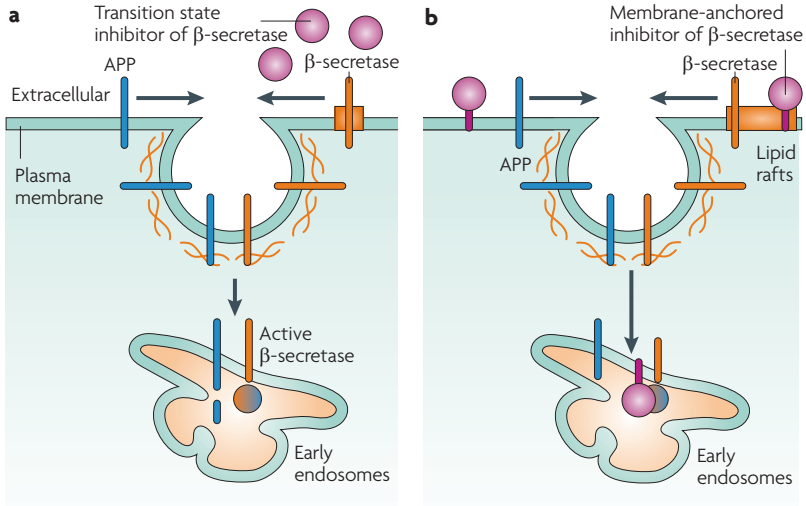


Figure 3 | **Endosomal targeting of β-secretase inhibitors.** β-secretase (shown as orange bars) is sorted from the plasma membrane to the early endosomes, in which it acquires an active conformation through endocytosis mediated by lipid rafts (shown as orange blocks) and clathrin (shown as orange strips). Activity of the enzyme is also dependent on its localization to lipid rafts. In early endosomal rafts, β-secretase cleaves amyloid precursor protein (APP), which initiates the production of the toxic β-amyloid peptide. As active β-secretase (when in an open conformation) is found only in early endosomes, transition state inhibitors that are not membrane permeant (shown as purple circles) are unable to gain access to these endosomes (**a**). Cholesterol linking enables membrane anchoring of the inhibitors (purple circles attached to the membrane), and subsequent membrane trafficking transports them to the endosomes to inhibit the endosomal raft-bound β-secretase (**b**).

compartments[51,84] (FIG. 4). Conjugating drugs to such toxins would therefore target them to the ER and eventually to the cytosol (discussed below). As peptide loading onto major histocompatibility complex I (MHC I) during antigen presentation occurs in the ER, peptides conjugated to Shiga toxin reach the ER through retrograde transport. Shiga toxin conjugates were also efficiently used for cross-presentation by MHC I and MHC II in dendritic cells[84], thereby conferring increased immunity against viral antigens. Interestingly, the receptor glycolipid for Shiga toxin B, globotriaosylceramide (GB3; also known as CD77), is overexpressed in many tumours. This makes GB3 a biomarker for detection of these carcinomas and presents an opportunity to target toxin conjugates of anticancer drugs and tracers used in tumour imaging to the tumour[85,86]. Topoisomerase inhibitors have successfully reached the ER when conjugated to Shiga toxin B subunit. The same strategy has been used to deliver the imino sugar *N*-butyldeoxynojiromycin to the ER, in which it inhibited the *N*-glycosylation of tyrosinase in melanoma cells[87].

Targeting to the cytosol

The cytoplasm hosts various metabolic, signalling and pathogenic processes that are targets for several diseases. Viruses deliver their genome to the cytosol by fusion of their envelopes at the plasma membrane or in endosomes. Post-transcriptional control mechanisms, such as those mediated by microRNA and small interfering RNA (siRNA), occur in the cytoplasm, and therapeutic siRNAs have to be targeted to this subcellular compartment (BOX 2). To target drugs to the cytosol and avoid lysosomal degradation, several strategies have been devised. These involve the use of cell-penetrating peptides (CPP) that permeate through the plasma membrane bilayer and release the drug directly into the cytosol; pH-responsive carriers that dispense the drug into the cytosol from the endosomes; and endosome-disrupting agents that aid in the release of drugs into the cytosol (FIG. 4).

CPPs that act directly at the plasma membrane. Direct transport of cargo across the plasma membrane can be achieved by conjugating molecules to CPPs[88]. The discovery that the HIV transactivator of transcription (Tat) protein can traverse cellular membranes and gain access to the nucleus stimulated considerable interest in exploiting this molecule for targeted drug delivery[89]. Several other proteins, including the transcription factor Antennapedia and the VP22 protein from herpes simplex virus[90], were also found to penetrate membranes. The peptidic regions responsible for cell penetration are either amphipathic (model amphipathic peptides or transportan) or arginine-containing stretches (Tat, VP22 or penetratin), usually ~30 amino acids long[88]. It is not clear how these peptides (and their conjugates of diverse chemical nature) cross the membrane at neutral pH, although interaction of cationic peptides with membrane phospholipids is thought to trigger a conformational change that allows their insertion into the membrane[91]. The mechanisms underlying the subsequent translocation through the lipophilic bilayers are also unclear. Emerging studies suggest that, although some peptides can traverse the membrane through lipid interactions, conjugates of CPPs are mainly internalized by endocytosis[92–94]. The uptake of labelled polyarginine occurred at spatially restricted membrane domains involving clathrin and dynamin[95]. In agreement with these findings, membrane anchoring of similar peptides through stearoylation or cholesterol modification increased the efficiency of cytosolic delivery by shifting the mode of internalization to endocytosis[95,96]. As CPP conjugates are internalized through endosomes and not directly to the cytosol, pH-sensitive drugs are likely to lose activity before reaching the cytosol. The direct translocation of the CPPs from the plasma membrane to the cytosol is evidently lost after conjugation and, despite enormous efforts, this delivery strategy is only successful in exceptional cases.

Peptide-mediated escape from early endosomes and the ER–Golgi network. To release the contents of endosomes into the cytosol, two strategies could be envisioned: fusion of a membrane-encapsulated particle or enveloped virus with the endosomal membrane, enabling the contents to be emptied into the cytosol (fusogenic mechanism); or disruption the endosomal membrane by an internalized particle, causing the endosome to discharge its toxic components into the cytosol (endoosmolytic mechanism)[97]. Viruses such as influenza use fusion mechanisms employing haemagglutinin protein. Under acidic pH conditions, haemagglutinin acquires

Small interfering RNA (siRNA). Small stretches of RNA, usually 21–25 nucleotides long, that bind to mRNA and target it for degradation, thereby silencing gene expression.

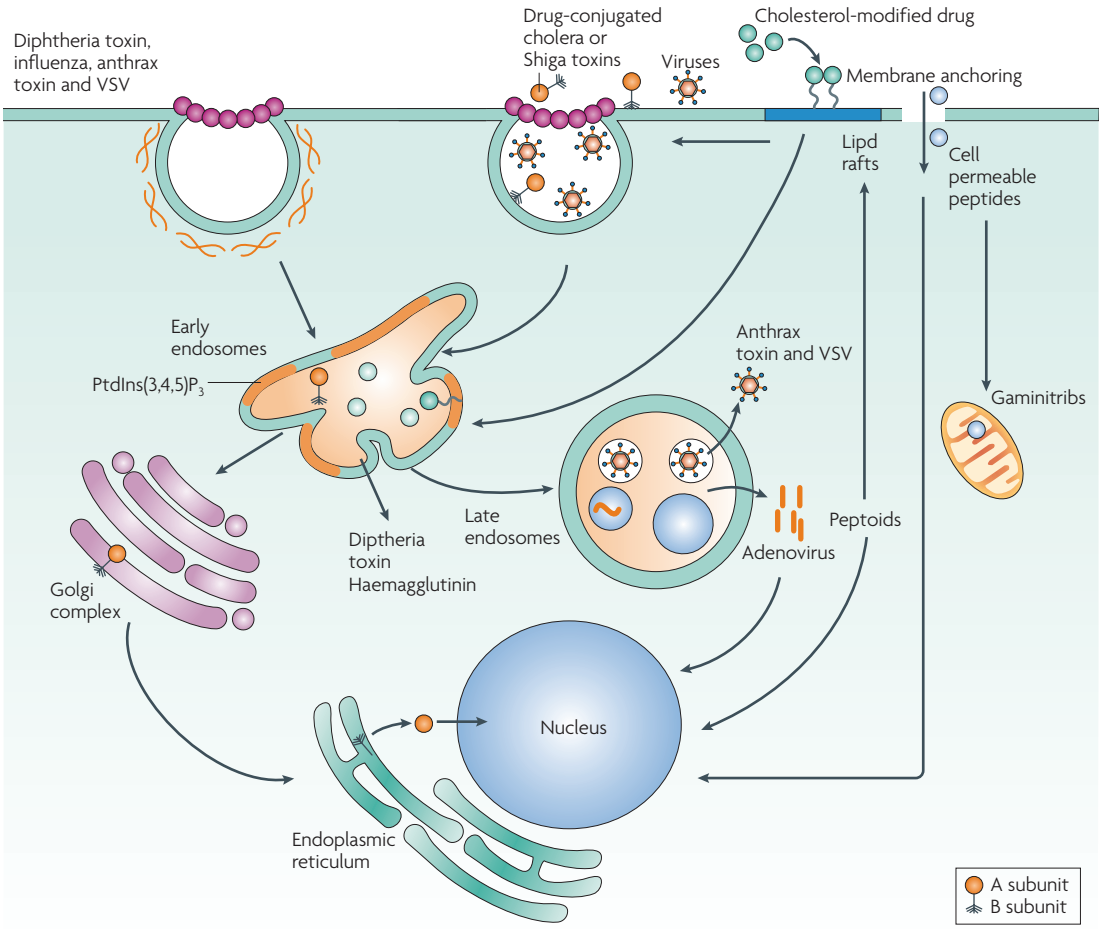


Figure 4 | **Strategies to target drugs to different cellular compartments.** Drugs conjugated to toxins such as diphtheria toxin are internalized through endocytosis mediated by clathrin (shown as orange strips) and are targeted to early endosomes. From here, the conjugated toxins are released into the cytosol through endosomal disruption. For some cargoes, such as anthrax or vesicular stomatitis virus (VSV), the release into the cytosol occurs through back-fusion of the intraluminal vesicles with the limiting membrane of the endosomes. Such release protects the conjugates or the toxins from the harsh degradative conditions of the endosomal lumen. Adenoviruses escape from endosomes at either the early or late endosomal stages, depending on the virus subtype. Once released into the cytosol, they are transported to the nucleus by motor-driven transport. Early endosomal transport of drug conjugates could be achieved by anchoring the drugs to cholesterol (shown as green circles). Cholesterol anchoring would target the drugs to lipid rafts (shown as blue bars) in the membrane. Cell-permeant peptides could directly traverse the plasma membrane and therefore localize to the cytosol. Gamitrinibs that contain cyclic guanidinium moieties target mitochondria whereas peptoids localize to the cytosol and nucleus. Cholera or Shiga toxins are sorted from the plasma membrane to the Golgi complex through the pentameric B subunit of the toxin. From there, the toxins are retrogradely sorted to the endoplasmic reticulum, in which the A subunit of the toxin is released into the cytosol. Drugs are conjugated to the B subunits of the toxins. PtdIns(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate.

a conformation that is able to insert into and fuse with the endosomal membrane, allowing the viral nucleocapsid to be released in the cytosol[98]. The amino-terminal region of the haemagglutinin protein of influenza contains a fusogenic peptide sequence, and several synthetic peptides have been designed to mimic the properties of these fusogenic sequences, including peptides containing GALA[99] or KALA sequences, which are used in the cytosolic delivery of conjugated drugs. In contrast to the fusogenic influenza mechanisms, adenoviruses and bacterial toxins use the endosomal membrane disruption mechanism[100]. Toxins such as diphtheria toxin or anthrax toxin are internalized into endosomes and undergo a conformational change that allows them to insert into the limiting membrane of early endosomes and escape the endosomes by rupturing the endosomal membrane[98]. Interestingly, recent evidence challenges these views and presents an alternative model of a fusion and release mechanism. In the case of anthrax toxin, the protective antigen is not directly released into the cytosol from endosomes, but is first delivered into the lumen of the intraluminal vesicles of the endosomes. Fusion of these intraluminal vesicles with the limiting membrane of the endosomes releases the internalized toxin into the cytosol (a process termed 'back-fusion')[101] (FIG. 4). This mechanism is proposed to further protect the toxin from

the harsh conditions in multivesicular bodies (MVBs), and a similar mechanism has been shown to mediate endosomal release of the nucleocapsid of vesicular stomatitis virus[102]. Polycationic polymers, such as polyhydroxyethyl aspartamide conjugated to histidine, also have pH-dependent endo-osmolytic properties. Such artificial polymers have been used to target doxorubicin to the nucleus by first releasing it into the cytosol from endosomes[103] for targeted cancer therapy.

Proteins can also be released to the cytoplasm from the ER. As part of the cell's 'quality control' checks, misfolded proteins are exported from the ER to the cytosol for degradation. This process is exploited by toxins such as cholera toxin to target their toxic A subunit to the cytosol. Immunotoxins or toxin conjugates are also used to traffic molecules to the cytosol[84,104] (FIG. 4).

Membrane anchoring of pH-sensitive probes has recently been shown to create novel prodrugs that increase the efficiency of cytosolic release[105]. Membrane anchoring of a pH-dependent lytic dodecapeptide and a fluorophore (5-carboxyfluorescein) can be achieved by linkage to sterols. In the case of the fluorophore, a disulphide-containing linker was used. The internalized fluorophore was confined to the endosomes as the endosomal lumen is predominantly in the oxidative state. However, following co-incubation with the cholesterol-anchored pH-dependent lytic peptide, the lytic peptide disrupted the endosomal membrane, allowing leakage of glutathione from the cytosol into endosomes. This influx reduced the disulphide bond in the cholesteryl-anchored fluorophore, enabling the release of the fluorophore into the cytosol. Inhibition of endosomal acidification by bafilomycin inhibited the release from the endosome[105]. Such prodrugs could be used to evade lysosomal degradation and to increase the efficiency of cytosolic release of molecules such as siRNAs[106–109].

By choosing appropriate lipid anchors and exploiting the large chemical space allowed in the linker region, organelle-specific targeting of inhibitors or prodrugs can be achieved (BOX 3).

Endosomal disruption and release of the luminal contents into the cytosol can also be triggered by photochemical internalization (PCI)[110]. PCI is based on the light-induced rupture of the endosomal membrane loaded with photosensitizing molecules. These photosensitizers are targeted to endosomes and, after they are internalized with the drug, photochemical illumination induces endosomal rupture through production of singlet oxygen and triggers the release of the internalized drug into the cytoplasm. However, this novel method has its limitations. The singlet oxygen that is produced could damage the conjugate itself, particularly in case of oligonucleotides, limiting the use of this technology.

Targeting to the nucleus

The nucleus contains various anticancer targets, ranging from proteins involved in DNA replication to nuclear receptors. DNA viruses have to deliver their genetic material into the host nucleus after gaining access to the cytosol, and studying virus entry has enabled the development of viral-mediated gene delivery[111]. One of the commonly used viruses for gene delivery is adenovirus[100] which, after endocytosis and rupture of the endosome, uses motor-driven translocation along microtubules to reach the nucleus. Binding of the nuclear localization signal in the viral capsid proteins to the nuclear pore is thought to mediate the entry of the virus.

However, some viruses, such as simian vacuolating virus 40 (SV40), mediate their entry into the nucleus by retrogradely trafficking from the plasma membrane to the ER followed by transfer to the nucleus[112]. Adenoviruses, adeno-associated viruses, retroviruses and lentiviruses have been used as delivery vehicles for gene therapy. However, owing to toxicity, immunogenicity and lack of specificity, these delivery methods are being replaced by specific viral machinery that lacks pathogenic components.

Viral and non-viral vectors for nuclear delivery. Small peptide regions from viruses that show nuclear translocation activity, such as the KKKRKV peptide derived from SV40 large T antigen, are used for the nuclear delivery of conjugated DNA. To avoid complications associated with viral-mediated delivery, non-viral vectors or chemical formulations that mimic the properties of the viruses have been designed to mediate gene transfer. These molecular formulations are often composed of lipids that form vesicles encapsulating the DNA molecule, such as the DNA molecules packaged into cationic liposomes, termed lipoplexes[113]. Polyplexes, by contrast, contain DNA in polymeric complexes comprised of polylysine, spermine or the dendrimer polymers, such as polyethylimine[97,114]. These polymers are internalized by various endocytic mechanisms, and incorporation of endo-osmolytic agents aid the cytosolic release of these conjugates[115]. Co-administration of peptides containing nuclear localization signals, covalent coupling or incorporation into lipid formulations are used to enhance nuclear delivery of conjugates. Finally, nuclear proteins such as histones and protamine that are highly enriched in basic amino acids can be used in gene delivery[100].

Delivery using nanoparticles. Nanoparticles are colloidal particles that are used as alternative delivery devices to liposomes or viral vectors[116–118]. Nanomedicine, a new field that is rapidly progressing, has shown promise in delivering drugs, some of which have already made it to the market[4,119]. In nanoparticles or nanocarriers, the drug or prodrug of interest is dissolved or encapsulated in the particulate matter[120]. These particles, either as suspensions or particulates, enhance stability and drug dissolution rate. Encapsulating with appropriate uptake signals might enable cellular uptake and specific targeting to subcellular destinations[121]. Whereas the smaller particulates could be taken up by fluid-phase endocytosis, the larger ones might be phagocytosed and targeted to phagosomes. Another issue in the subcellular delivery of nanoparticles is the release into the cytosol. Exploiting the lower endosomal pH compared with the cytosol, pH-sensitive polymers could be designed to enable the release of the drugs from the particulate material.

Singlet oxygen
A form of molecular oxygen that is a reactive oxygen species and less stable than the normal triplet oxygen.

Box 2 | **RNAs and lipid modifications**

Cytosolic drug targeting without major losses to endolysosomes is desired when designing small interfering RNA (siRNA) conjugates for therapy[109]. To promote delivery, RNAs are either encapsulated in lipid vesicles or conjugated to membrane-penetrating peptides. Stable nucleic acid–lipid particles (SNALPs) or interfering nanoparticles carrying siRNAs have been designed to deliver siRNAs to target organs *in vivo*. For example, SNALPs containing siRNAs targeting hepatitis B virus (HBV) RNA effectively inhibit the replication of HBV[162]. Similarly, by coupling with N-acetyl-galactosamine and disulphide-linked endo-osmolytic polyvinylether, which facilitates hepatocyte targeting, endocytic uptake and cytoplasmic release of siRNAs through endo-osmolysis was achieved[107]. This technology, termed dynamic polyconjugation, is used to effectively silence the genes encoding low-density lipoprotein (LDL) and peroxisome proliferator-activated repetor-α in mouse liver. Recently, β1,3-D-glucan-encapsulated siRNA particles (GERPs) were used as efficient oral delivery vehicles for silencing genes in mouse macrophages[163].

Targeting can also be achieved by covalent attachment of siRNA to cholesterol and other lipids[108]. Recently, siRNAs against apolipoprotein B (APOB) were modified with cholesterol and were shown to reduce APOB mRNA levels[164]. This reduction occurred several fold more efficiently when cholesterol–siRNAs were associated with high-density lipoprotein (HDL) particles. The resultant decrease in the levels of plasma LDL and serum cholesterol underscores the possibility of such modifications for therapeutic use. Other lipophilic conjugates such as fatty acids and bile acids have also been shown to aid the delivery of siRNAs. siRNAs conjugated with long-chain fatty acids such as stearoyl (C18) and docosanyl (C22) silenced the *APOB* gene more efficiently than their shorter counterparts such as lauroyl (C12) and myristoyl (C14) chains. Higher lipophilicity, which is conferred by the longer saturated chain lengths, seems to determine the affinity of these conjugates for HDL and subsequent efficiency of gene silencing[164]. As different lipid anchors partition into different lipoproteins, this property could also be exploited to transport conjugates to different target organs. Despite these advances, this promising drug strategy requires improved tissue delivery *in vivo* to become more generally applicable.

Nuclear delivery through peptoids. Peptoids are a novel class of peptidomimetics that penetrate cells mainly through a backbone containing five or six glycine residues[122]. Two kinds of peptoids have been designed, one with amino side chains and the other with guanidinium side chains[122]. Both types are positively charged but are sorted differently. The amino-modified peptoid is targeted to the cytoplasm whereas the peptoid with the guanidinium side chain is trafficked to the nucleus. This indicates that the nature of the side chain determines the uptake kinetics and destination of the peptoid, potentially facilitating the design of drugs that target distinct compartments.

Drug targeting to mitochondria

Drugs reaching the cytosol need to be further targeted if their targets reside in membrane-bound compartments that are not accessible from the surface by endocytic routes. Mitochondria serve as hotspots for targeted therapy both in host cells and in parasites. In host cells, mitochondrial proteins such as B cell lymphoma family proteins serve as anticancer targets. Inhibiting the electron transfer chain or the redox system in parasite mitochondria is a successful antimicrobial approach[123]. Furthermore, dysfunction in mitochondria has been observed in several neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis.

Drugs that could be specifically targeted to mitochondrial proteins are therefore of therapeutic interest. CPPs derived from mitochondrial proteins, such as cytochrome oxidase subunits and gene associated with retinoic-interferon-induced mortality (Grim) proteins, have been shown to target heterologous proteins, peptides and small molecules specifically in mitochondria[124]. Lipophilic cationic conjugates of anticancer agents target mitochondria to trigger cell death in target tissues[125–127]. A novel class of aromatic cationic Szeto–Schiller peptides selectively partition into the inner mitochondrial membrane and confer mitoprotection through their antioxidative properties[128]. These peptides readily cross the blood–brain barrier and reduce the production of mitochondrial reactive oxygen species, and are showing promise for the treatment of neurodegenerative diseases. Recently, a novel class of compounds termed gamitrinibs (so called because they are geldanamycin mitochondrial matrix inhibitors) was shown to exclusively target mitochondrial heat shock protein 90 (HSP90) in tumour cells[129]. In mitochondria, HSP90 plays an active part in many tumours, and inhibitors of HSP90 such as geldanamycin have been shown to have antitumour properties[130]. As these inhibitors also show inhibitory activity to HSP90 and other heat shock proteins located in the cytosol, specific targeting of these inhibitors to mitochondria could increase efficacy and reduce toxicity. Gamitrinibs contain geldanamycin as the active molecule, linked through an amide-containing linker to cyclic guanidinium moieties, which act as mitochondrial targeting signals. Only those containing three to four units enabled efficient intracellular uptake and mitochondrial targeting in a cellular context (FIG. 4). Surprisingly, no mitochondrial toxicity in non-tumour cells was reported, making gamitrinibs attractive novel antitumour drugs[130].

Compartmentalization of RNA and lipids

In addition to proteins, lipids and RNA molecules have been shown to be localized to distinct subcellular locations[131]. Lipids such as phosphoinositol phospholipids (PIPs) exhibit polarized localization in the epithelial cells, and different PIPs are localized in different organelles (FIG. 2). These lipids play an important part in cell polarity, migration and in pathological conditions[131–133]. Sphingolipids such as ceramides are selectively recruited to raft domains in the membrane and initiate apoptotic signals[9]. Ceramide recruitment to early endosomes is important for the formation of intraluminal vesicles of the exosomal subset of MVBs[134]. Whether site-specific modulation of these lipids can be achieved by subcellular targeting approaches is yet to be experimentally demonstrated.

Spatially restricted protein synthesis occurs by recruiting mRNA to subcellular compartments[135]. Recent studies have shown that a large number of mRNAs localize to different subcellular sites, such as centrosomes, apical or basolateral domains, spindle poles or axon dendritic compartments[136]. These studies also suggest that mRNA localization is crucial for the formation of functionally distinct compartments[135]. Recent studies show that, in addition to mRNAs, regulatory RNAs such as micro RNAs (miRNAs) are also localized in spatially distinct sites in

Blood–brain barrier
A semi-permeable cellular structure consisting of endothelial cells that allows selective passage of some molecules but prevents the passage of others.

Box 3 | **Designing tripartite inhibitors for subcellular targeting**

Covalent linkage of lipids to drugs could be achieved in several ways depending on the nature of the drug and the lipid. Modification should preserve drug function and should not affect the partitioning and trafficking of the lipid. Drugs are often conjugated to a lipid through a linker, giving rise to 'tripartite' molecules (see the figure).

The pharmacophore (the 'message')

The main part in the tripartite molecule is the active ingredient (the drug) against a target molecule that is localized in a particular subcellular space. The pharmacophore could also be designed as a prodrug that exposes the active moiety in the appropriate environment. The pharmacophore could include drugs in combination with imaging dyes or moieties that couple the pharmacophore to the linker.

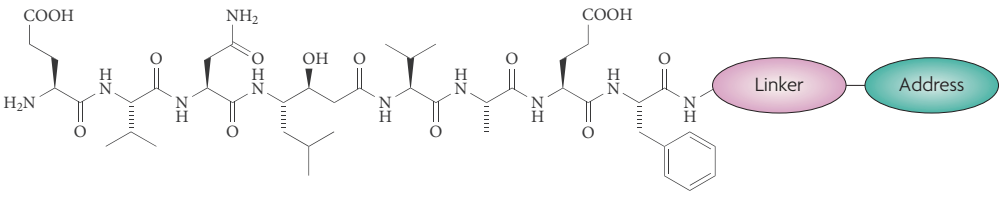
The lipid anchor (the 'address')

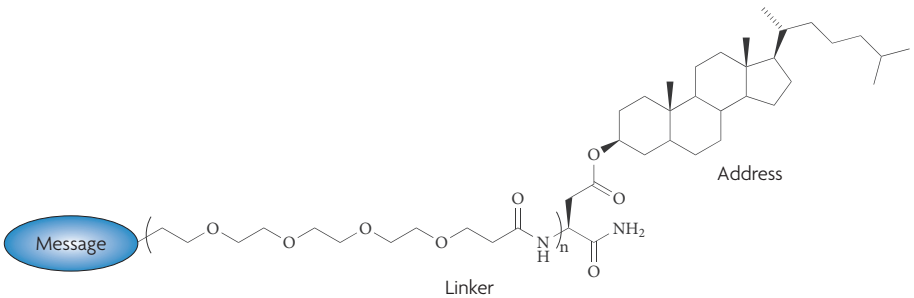
The lipid tails anchored to the drug determine their sorting in subcellular compartments. Lipids differ in their membrane-partioning properties and in their sorting behaviour[165]. For example, cholesterol is more readily taken up in biological membranes than long-chain fatty acids. However, it is trafficked mainly through lysosomes, in which it can be degraded, affecting the stability of the linked molecule. By carefully choosing various lipids as anchors, it could be possible to sort the conjugates to different subcellular and submembrane compartments such as lipid rafts[34,166]. As lipid-raft domains contain several drug targets, lipids that are enriched in these regions (such as cholesterol and sphingolipids) could be used to target drugs to these domains. Cholesterol and sterol analogues such as ergosterol, stigmasterol, dihydrocholesterol, sphingolipids, gangliosides, globosides, ceramides and sulphatides could be used as lipid anchors[167,168]. For targeting drugs to other submembrane compartments, phospholipids, unsaturated fatty acids and glycerophospholipids could be used. The fatty-acid composition and chain length of globotriaosylceramide (GB3), the receptor glycolipid for Shiga toxin B, have been shown to regulate sorting of the toxin to the Golgi complex[34]. Particular fatty-acyl moieties have also been reported to mediate the retrograde trafficking of two classes of verotoxins[169,170]. Sphingomyelins that vary in their fatty-acyl chain lengths are sorted to different compartments in the cell: long-chain, ordered-domain-preferring (or raft-partitioning) sphingolipids are targeted to late endosomal compartments in a cholesterol-dependent manner, whereas short-chain, disordered-domain-preferring lipids are recycled more effectively independently of cholesterol levels[171]. These findings highlight the importance of selecting the appropriate lipid chains to modulate the kinetics of internalization and directing of drugs to various subcellular destinations.

Important considerations when selecting a lipid anchor include: a high level of partitioning into biological membranes; high micellar concentrations; easy transport into the bloodstream by lipoproteins or lipid-binding proteins; low non-specific adsorption to the tissues at the site of injection; and faithful targeting of the conjugates to the respective subcellular destinations.

The linker

A linker, which is usually an oligomer, is introduced to connect the active pharmacophore to the lipid anchor. Direct conjugation of the drug to the lipid molecule might hinder the interaction of the drug with its intended target by causing steric hindrance or by placing the drug too close to the lipid bilayer. Oligo(ethylene glycol) or oligoamides can be used as backbone structures for linkers[105,172]. The linker lengths could be adjusted to optimize accessibility to the target molecule. In some cases, the optimal length of the linker could be designed by analysing the drug–target interaction site and the distance of this interaction site from the bilayer. Linkers also provide space to introduce additional modifications, such as sites for enzymatic cleavage, pH-dependent cleavage moieties, disulphide bonds to modulate glutathione-dependent reduction and consequently the liberation of linked molecules. Such linkers are crucial for the design of lipid-linked prodrugs[105].





the cell. Using expression profiling of miRNAs enriched in synaptosomes, miRNA-138 was found to negatively regulate dendritic spine size in rat hippocampal neurons[137]. Both miRNAs and siRNAs are now known to require membrane trafficking through functional MVBs[138,139]. mRNA silencing through siRNAs is increasingly being used for disease intervention, and targeting therapeutic RNA interference drugs to these compartments might enhance their efficiency[140].

Outlook

Drug delivery remains a considerable challenge in effective drug design. In this context, an understanding of how cells traffic their constituents to the correct location inside or outside the cell could provide valuable information. Cell biological strategies that take advantage of cellular distribution systems have so far not had the role in drug development that they deserve. Usually, drugs are designed to overcome the cellular barriers that impede access to their targets. The consequences are often increased toxicity and therapeutic failure. Can we improve drug specificity by increasing its concentration in the cellular compartment in which the drug exerts its action? This is an important field to be explored. Ongoing studies that are trying to exploit cellular machineries for specific delivery are showing promise; these are examining transport in extracellular vehicles such as exosomes and lipoproteins, the crossing of the blood–brain barrier by transcytotic routes and the addition of targeting tags and lipid moieties to facilitate cellular delivery[141–143]. There are a myriad of possibilities yet to be tried.

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Acknowledgements
We thank M. Zerial, I. Levental, J. Ries, V. Kotelianski, C. Schroeder, Z. Maliga, G. Kalyanaraman and G. Jennings for their insightful comments on the work. We acknowledge the funding support from the Swiss National Foundation (NCCR Neuro) and a grant from Alzheimer Forschungs Initiative e.V to L.R.

Competing interests statement
The authors declare competing financial interests: see web version for details.

DATABASES
OMIM: http://www.ncbi.nlm.nih.gov/omim
Alzheimer's disease | amyotrophic lateral sclerosis | Niemann–Pick disease type A | Niemann–Pick disease type B | Niemann–Pick disease type C | Parkinson's disease
UniProtKB: http://www.uniprot.org
CDC42 | HSP90 | PAK1 | PAK2 | RAB4 | RAB11 | RAC1

FURTHER INFORMATION
Lawrence Rajendran's homepage: http://www.neuroscience.ethz.ch/research/aging_disorders/rajendran
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