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ALTERNATE ROUTES FOR DRUG DELIVERY TO THE CELL INTERIOR:

PATHWAYS TO THE GOLGI APPARATUS AND ENDOPLASMIC RETICULUM

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Abstract

The targeted delivery of drugs to the cell interior can be accomplished by taking advantage of the various receptor-mediated endocytic pathways operating in a particular cell. Among these pathways, the retrograde trafficking pathway from endosomes to the Golgi apparatus, and endoplasmic reticulum is of special importance since it provides a route to deliver drugs bypassing the acid pH, hydrolytic environment of the lysosome. The existence of pathways for drug or antigen delivery to the endoplasmic reticulum and Golgi apparatus has been to a large extent an outcome of research on the trafficking of A/B type-bacterial or plant toxins such as Shiga toxin within the cell. The targeting properties of these toxins reside in their B subunit. In this article we present an overview of the multiplicity of pathways to deliver drugs intracellularly. We highlight the retrograde trafficking pathway illustrated by Shiga toxin and Shiga-like toxin, and the potential role of the B subunit of these toxins as carriers of drugs, antigens and imaging agents.

Keywords

A/B toxins; caveolae; endoplasmic reticulum; Golgi apparatus; lipid rafts; lysosomes; receptor-mediated endocytosis; retrograde-trafficking pathway; Shiga/Shiga-like toxin; targeted drug delivery

1. Overview

Targeted drug delivery is a major goal of chemotherapy. It both maximizes target effect and minimizes collateral damage. The achievement of this goal is clearly a significant challenge. It requires detailed knowledge of cells and their properties and the development of effective drug targeting strategies that are likely to be specific to each individual case. Moreover, it requires not only targeting the right cell, but also the intracellular delivery of the drug. The cell membrane or plasma membrane is a barrier for many molecules, and particularly for charged macromolecules such as the proteins or peptides that are often used as targeting vehicles for drugs and the oligonucleotides that may become popular in small interfering RNA (siRNA) based therapies [1]. In many cases, drugs need to gain entrance into the cell in order to exert their action, and within cells a specific localization may be required, cytosol, nucleus, mitochondria, the secretory pathway, etc. The membrane must be crossed. Furthermore, the

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chemical stability of the drug must be maintained during transit to its intracellular site of action. Typically, many protein-targeted drugs are taken up through endocytosis and are then delivered progressively to early endosomes, late endosomes and eventually to lysosomes where they likely may be degraded. Lysosomal delivery, however, can be avoided or minimized. In this article, we examine the range of choices that may be taken to minimize the exposure of drugs to the acid pH, hydrolytic environment of the lysosome. In doing so, we focus first on the opportunities for alternative intracellular delivery that nature provides and then discuss examples of strategies that have been employed to date. As we proceed, we highlight pathways for delivery of drugs to the Golgi apparatus (GA) and endoplasmic reticulum (ER), key organelles of the secretory pathway. Proteins are synthesized in the endoplasmic reticulum and transported to the Golgi apparatus where they are modified and sorted before secretion. We close by offering some perspective on future strategies that may be taken.

2. THE BIOLOGICAL SMORGASBORD OF RECEPTOR-MEDIATED INTRACELLULAR PATHWAYS FOR POSSIBLE DRUG DELIVERY

The internalization of ligand/receptor complexes at the plasma membrane can occur by several mechanisms. The best-studied endocytic mechanism involves the formation of clathrin-coated vesicles. More recently discovered clathrin-independent mechanisms have been described and are less well understood. The clathrin-independent pathways include endocytic mechanisms initialized within lipid-rich microdomains at the plasma membrane, represented by lipid rafts- and caveolae-dependent uptake. These endocytic mechanisms are illustrated in Figure 1. Macropinocytosis and phagocytosis are also clathrin-independent pathways [2]. Macropinocytosis holds little, if any, promise for cell targeted delivery and hence will not be covered further in this review. Phagocytosis can be cell targeted. However, it concerns the uptake of large sized particles and has not been the subject of targeted drug delivery yet.

2.1 Clathrin-dependent uptake

Clathrin-dependent uptake is the best-defined endocytic mechanism. Clathrin is a cytosolic fibrous protein with the shape of a three-limbed triskelion that has the ability to self-polymerize into a basket-like structure, typical of the ones observed in clathrin-coated vesicles [3]. Biogenesis of clathrin-coated vesicles starts by recognition of specific targeting sequences in the cytosolic domain of plasma membrane receptors by cytosolic adaptor proteins. The most common endocytic signals encompass tyrosine-based and leucine-based tetrapeptide sequences that interact with several cytosolic adaptor proteins, principally AP-2 (adaptor/assembly protein 2), AP180, Eps 15 and Epsin [4-6]. Adaptor proteins are involved in the location of clathrin assembly on the plasma membrane and promotion of clathrin polymerization. Initially, adaptor proteins Epsin and AP-2, besides interacting with the receptor signal motifs, bind regions of the inner leaflet of the plasma membrane rich in phosphatidyl inositol, 4,5 bisphosphate [$PI(4,5)P_2$] with subsequent recruitment of other adaptor proteins, clathrin and assembly of the clathrin coat [7,8]. Recruited clathrin molecules assemble within the plasma membrane into a planar lattice that further develops into the cage/basket-like structure with concomitant formation of a deeply invaginated clathrin-coated pit that leads to the release of the clathrin-coated vesicle [9,10]. Apparently the curvature of the clathrin-like basket structure provides the driving force for the formation and release of the vesicle from the plasma membrane, together with dynamin, a protein that binds and hydrolyses GTP [3, 11]. Interestingly, it has been shown that dynamin is involved in actin filament assembly, as well as that dynamin and actin are recruited sequentially at sites of endocytosis [12,13]. Cholesterol may also contribute to membrane invagination since cells depleted of cholesterol with sterol-binding agents, show numerous flat clathrin-coated patches at the plasma membrane, and very little deeply-invaginated coated pits [3,14,15].

Clathrin-dependent endocytosis is the starting point for the transport of ligand to early or sorting endosomes where proteins and lipids to be reused are salvaged in recycling endosomes and returned to the plasma membrane. The remaining proteins and lipids are transported to late endosomes and in turn to lysosomes where they are degraded. However, this intracellular pathway is one pathway among multiple ones co-existing in a cell [16]. For example, A/B toxins such Shiga toxin (ST), Shiga-like toxins (SLT), Ricin, Cholera toxin (CT), *E coli* heat labile toxin (HLE), and *Pseudomonas* exotoxin (PE) can enter the cell through clathrin-dependent or -independent endocytosis but move from early endosomes directly to the GA, sidestepping the late endosome/lysosomal path and hence avoiding degradation.

2.2 Clathrin-independent uptake: lipid rafts and caveolae

Lipid rafts have been defined as plasma membrane microdomains with a highly ordered structure distinct from the surrounding membrane area, rich in cholesterol, glycosphingolipids, and saturated lipids [17]. The lipid rafts are thought to comprise both leaftlets of the plasma membrane. Glycophosphatidylinositol (GPI)-anchored proteins are present in the exoplasmic layer of the plasma membrane while palmitoylated and myristoylated proteins are in the cytoplasmic phase, as well as many other membrane proteins in both sides of the membrane. Lipid rafts were originally characterized as plasma membrane fractions insoluble in cold Triton X-100, a non-ionic detergent, and sensitive to cholesterol depleting agents [17,18]. Proteins found in these detergent-insoluble membranes besides the GPI-anchored proteins, include many acylated signaling proteins (e.g. Src family kinases), growth factor receptors, G-proteins, nitric oxide synthase, integrins, and cholesterol-binding proteins such as caveolin. Also signaling lipids as for example, $PI(4,5)P_2$, arachidonic acid and phosphatidyserine are present in the lipid rafts [19]. It is thought, that lipid rafts may have a role in the segregation of signaling proteins and lipids within a membrane microdomain to increase proximity, efficiency, specificity and regulation of signaling cascades [20].

A specialized sub-type of lipid rafts is caveolae. Caveolae are uncoated cell surface invaginations first observed by electron microscopy by Palade [21] and Yamada [22,23] more than half a century ago. Caveolae are not present in all cell types, for example they are abundant in muscle, endothelia and adipocytes corresponding to about 35% of the cell surface, in contrast they are absent in lymphocytes and neurons [17]. Caveolae are also characterized by the presence of the protein caveolin, and similar lipid and protein content as lipid rafts [24-26]. Caveolae thus colocalize with detergent-insoluble membrane fractions, are sensitive to cholesterol depletion and share many of the molecular markers present in lipid rafts [24-29]. Caveolin is a 21 kDa integral membrane protein, with 3 covalently-attached palmitoyl groups, that binds cholesterol [17,28]. Caveolin has an atypical topology in the plasma membrane as it forms an intra-membrane hairpin loop, with both N- and C-terminal domains in the cytosol [30]. Three isoforms of caveolin have been described, caveolin-1 and caveolin-2 present in all cells with caveolae, and caveolin-3 found only in muscle cells [17]. The role of caveolae and lipid rafts in endocytosis has been surrounded by controversy since several studies have indicated that in caveolae-containing cells, only a minor pool of caveolae is engaged in active endocytosis. This was best illustrated by fluorescence recovery experiments after photobleaching (FRAP) [31] in cells expressing chimeric caveolin-1-green fluorescent protein (GFP) in which they found that caveolae labeled with the caveolin-1-GFP remained static. Moreover, in this study they found that integrity of caveolae is dependent on cholesterol as treatment with methyl-β-cyclodextrin, a cholesterol-depleting agent, caused disappearance of caveolae structures and redistribution of caveolin-1 along the plasma membrane. Likewise, treatment with cytochalasin D, which causes actin cytoskeleton depolymerization, resulted in clustering of the caveolae at the plasma membrane, indicating a role of the actin cytosckeleton in stabilization of caveolae at the plasma membrane [31,32]. Other lines of evidence have shown that filamin, an actin binding protein, is a ligand for caveolin-1, and activation of Rho,

which causes reorganization of actin cytoskeleton, also affects redistribution of caveolae [33]. Moreover, treatment of cells with okadaic acid, a phosphatase inhibitor, stimulates caveolae-dependent endocytosis, and genistein, a Src-family kinase inhibitor, prevents caveolar endocytosis, indicating that reversible phosphorylation may play a role in caveola formation and budding [29,34]. The nature of this effect is unknown [29]. Other studies have shown that overexpression of caveolin-1 in transformed NIH-3T3 cells decreases the rate of endocytosis of the autocrine motility factor receptor (AMF-R), a transmembrane protein with steady-state residency in caveolae at the plasma membrane and the smooth ER, indicating that caveolin-1 may actually be a negative regulator of caveolae-dependent endocytosis [35]. Consistent with this is the fact that transformed NIH-3T3 with low-level expression of caveolin-1 showed greater caveolar-dependent endocytosis compared to the cells overexpressing caveolin-1 [35]. As in clathrin-dependent endocytosis, caveolae-dependent endocytosis relies on the GTPase dynamin (see above), which may function to regulate the budding of caveolae from the plasma membrane [27,35].

Extensive studies on Cholera toxin (CT) and simian virus 40 (SV40) internalization have demonstrated the involvement of caveolae in their endocytosis. SV40 infects cells through binding to major histocompatibility class I (MHCI) antigens at the cell surface. Pelkmans et al. [36] have demonstrated that SV40 enters CV-1 cells (African green monkey kidney fibroblast cell line) by caveolae using dual color live fluorescence microscopy with Texas Red-labelled virus and GFP-caveolin-1 and tubulin. They observed that SV40 associates with caveolae at the plasma membrane and moves from the plasma membrane in small caveolin-1-positive vesicles into caveosomes, a neutral pH compartment that does not contain markers of endosomes, lysosomes, ER or GA. SV40 is transported from caveosomes directly to smooth ER using caveolin-1-free tubular vesicles that move along microtubules (see Figure 1, pathway *A*). Additionally, SV40 binding to its receptor in caveolae induces actin cytoskeleton reorganization and recruitment of dynamin [37]. SV40 infection is also dependent on cholesterol and activation of tyrosine kinases in caveolae [37]. The pathway of entry of SV40 is unusual in that it initially enters the caveosomes, an organelle biochemically distinct from endosomes, thus bypassing endosomes and GA to reach the smooth ER. Surprisingly, in a recent study, it was found that SV40 can infect cells that do not express caveolin-1, initially transferring to neutral pH caveosome-like structures devoid of caveolin-1 and then transferring to the ER via microtubule-dependent vesicular carriers [38]. This alternative pathway is independent of caveolae, clathrin, dynamin, and Arf6 (small GTPase), but dependent on cholesterol and tyrosine kinases activation [38]. The autocrine motility factor receptor (AMF-R) (see above), a cellular protein in NIH-3T3 cells, upon binding AMF is internalized via caveolae-, dynamin- and chlolestol-dependent endocytosis, and subsequently taken directly to the smooth ER by vesicular/tubular transport, in a similar fashion as SV40 [35], suggesting that the SV40 intracellular route to the ER may actually be a normal membrane trafficking pathway.

Cholera toxin (CT) can enter the cell by multiple endocytic pathways, it can enter through clathrin-mediated endocytosis as well as by clathrin-independent mechanisms [39-42] (see Figure 1 pathways *A1*, *B*, *C*). Within the clathrin-independent mechanisms CT can enter either by caveolae or lipid rafts [39-44]. The receptor for CT is GM1, a glycosphingolipid associated with lipid rafts and caveolae [44,45]. In fact, GM1 is generally used as a marker for lipid rafts. CT has been shown to be internalized via caveolae in a dynamin and cholesterol dependent manner [46,47]. Nevertheless, it is also true that in cells lacking caveolae, CT is able to enter cells through association with lipid rafts, even in cells expressing dominant negative mutants of dynamin [42]. This caveolae/dynamin-independent pathway is sensitive to cholesterol depletion, pointing out the contribution of lipid rafts in the process. Following internalization by clathrin dependent or independent-endocytosis, CT collects in early endosomes, although it was shown that CT taken up via caveolae can localize to caveolin-1 positive compartments

(caveosomes) [41] (see Figure 1, pathways *A1*, *B*). Interestingly, in a recent report [48], it was shown that SV40 can actually bind GM1, the receptor for CT, and promotes direct transport to ER. There is no evidence though that CT uses this direct path to ER.

Shiga toxin (ST) and Shiga-like toxin (SLT) can also enter cells by clathrin-dependent and independent pathways [49-51] as illustrated in Figure 2. The receptor for ST/SLT is the glycosphingolipid Gb_3 that can also localize to lipid rafts. ST/SLT can be internalized by clathrin-independent endocytosis mediated by association to lipid rafts, and seemingly independent of the GTPase dynamin [49]. For ST/SLT there is no evidence to support caveolae-mediated uptake [16]. Subsequent to uptake by clathrin-dependent or -independent pathways, ST/SLT initially localizes to early endosomes [52].

In conclusion, these pathways can offer a route for drug delivery to the Golgi apparatus and endoplasmic reticulum that bypasses degradative compartments.

2.3 Bypass transport from endosomes to GA and ER

Most of the A/B family of protein toxins once they have penetrated the cell by clathrin-dependent or -independent mechanisms, usually accumulate in the early endosomes, as demonstrated by co-localization with markers of this compartment, e.g. transferrin receptor, early endosome antigen 1 (EEA1). Some of these toxins such as Diphtheria, *Chlostridium*, and *Anthrax* toxins, continue transport to the more acidic late endosomes (see Figure 1, pathway *D*) where their toxic A subunit can translocate into the cytosol. These toxins will not be covered here, for reviews on this topic, see Cabiaux [53], and Watson and Spooner [54]. Alternatively, CT, ST/SLT, PE, HLE and ricin translocate into the cytosol from the ER, but in order to reach the ER they pass by the trans Golgi network (TGN), a trans Golgi subcompartment, and the Golgi apparatus in total, in retrograde fashion (see Figure 1 and Figure 2). This retrograde route was discovered and delineated by studies done primarily with ST/SLT [52,55,56], accordingly, we will use ST/SLT as an example to illustrate this intracellular route. The initial assumption was the ST/SLT once in early endosomes would move to late endosomes and move along the well-established path taken by the mannose-6-phosphate receptor (M6PR) to recycle back to the Golgi apparatus. The M6PR binds lysosomal proteins in TGN and targets them to the lysosomes via endosomes [57]. M6PR is retrieved from late endosomes back to the TGN and reused to deliver more lysosomal enzymes to lysosomes (see Figure 1, pathway *D*). The transport of M6PR from late endosomes to GA is regulated by the small GTPase, Rab9 [58]. Expression of inducible Rab9 dominant negative mutant in cells, inhibits transport of M6PR to TGN but does not inhibit localization of ST/SLT and ricin to TGN, indicating that ST/SLT and ricin do not transit along with M6PR to TGN [59-61] (see Figure 2). In addition, in cells that internalize ST/SLT by clathrin-dependent endocytosis, clathrin is also required to deliver ST/SLT to TGN, as well as the adaptor protein epsin, dynamin, Rab11, Rab6A' and cholesterol [49,51,52,62-66]. Molecular fusion machinery involved in the transport of ST/SLT to TGN include the v-SNAREs, VAMP3/cellubrevin and VAMP4, and the t-SNAREs, syntaxin 6, syntaxin 16, and Vti1A [62,65]. Other SNAREs like syntaxin 5, GS28, Ykt6, and GS15 have also been reported to mediate transport of ST/SLT to TGN [67]. Additionally, cytosolic Ca^{+2} concentration seems to affect delivery of ST/SLT to TGN, as increased Ca^{+2} accelerates transport of ST/SLT as compared to normal conditions [61].

Once ST/SLT reaches the TGN (also CT, HLE, PE, and ricin) it moves to the ER in a COPI-independent retrograde transport (see Figure 2). COPI is a coat complex with a function similar to that of the clathrin coat [4,68-72]. COPI-coated vesicles are involved in anterograde vesicle traffic within the GA and in retrograde traffic from GA to ER to retrieve ER resident proteins that escape the ER. COPI coat components recognize a tetrapeptide sequence (KKXX or RRXX) in the cytosolic domain of ER resident proteins that act as retrieval signals [4,70,71]. The ER luminal proteins that escape to GA are retrieved by the KDEL receptor, which

recognizes the KDEL amino acid sequence in luminal proteins, and in turn the KDEL receptor is recognized by the COPI coat. The KDEL receptor system actively operates in the GA and ER/GA intermediate compartments to sort out and capture escaped ER resident proteins. The retrograde transport of these toxins should be mediated by the KDEL receptor system. ST/SLT does not have a KDEL signal, moreover addition of a KDEL signal to Shiga toxin subunit B (STB) does not improve its rate of retrograde transport to ER [73]. CT has a KDEL sequence in the catalytic A subunit, however, the CT B subunit by itself is able to reach the ER without the A subunit [74]. HLE has a KDEL-like signal in the toxic A subunit (RDEL), that likewise is not absolutely necessary for transport to the ER [74]. PE has a KDEL-like signal too, RDELK that itself does not bind the KDEL receptor, but the last lysine is removed during internalization, thereby, creating the RDEL sequence that binds the KDEL receptor [75]. The RDEL sequence seems to be necessary for PE to reach the ER, however, recent studies indicate that PE may be able to use more than one pathway to reach the ER [76].

Then, how do these toxins reach the ER? In recent years it has been established that GA resident enzymes constantly cycle from the GA to ER and back. The continuous cycling of GA resident enzymes has been demonstrated by experiments in which treatment with brefeldin A (BFA) causes re-localization of GA resident proteins to the ER, and practically the collapse of the GA into the ER [68,72,77,78]. BFA is a fungal macrolide that inhibits Arf1, a small GTPase necessary in the recruitment of COPI coat component proteins to GA membranes, and thus blocks COPI-dependent anterograde and retrograde transport from GA to ER. Given that retrograde traffic from GA to ER, independent of COPI, continues, the outcome is relocation of GA lipids and resident proteins to the ER and dispersal of the GA. Similarly, expression of dominant-negative acting mutants of Arf1 or components of the COPI coat complex [68,72, 79-81], as well as expression of dominant-negative acting mutants of other small GTPases involved in ER to Golgi membrane traffic such as Sar1p, a small GTPase required for recruitment of COPII coat proteins to the ER membranes [81-86], Rab6 [79,87-90], Rab33b [90,91], and other Rab proteins, all cause the same effect as that of BFA treatment, redistribution of GA proteins and lipids to ER. Girod, Storrie et al. [79] have reported that microinjection of anti-COPI coat antibodies or expression of Arf1 mutants does not interfere with GA to ER transport of ST/SLT or with the apparent recycling to the ER of GA resident proteins. In contrast, overexpression of a negative dominant mutant of Rab6 (Rab6-GDP), a small GTPase involved in intra GA transport, blocks transport to the ER of ST/SLT and GA glycosylation enzymes, but not the KDEL receptor or PE, a toxin containing a KDEL-like retrieval signal. This evidence supports the existence of an alternate transport pathway from GA to ER, independent on COPI but dependent on Rab6, that normally operates in the cycling of GA enzymes but exploited by toxins to reach the ER where they translocate into the cytosol to exert their toxic effect (see Figure 2). Transfer of the toxic catalytic A subunit of these toxins from the ER to the cytosol is thought to occur through the ER-associated protein degradation (ERAD) machinery. The ERAD machinery translocates misfolded proteins into the cytosol through the Sec61p translocon, for ubiquitination and subsequent degradation by the proteasome. CT, ricin, PE and ST/SLT have been shown to interact with Sec61p translocon components, indicating that these toxins takeover the cellular ERAD machinery for retrotranslocation from ER into the cytosol [92-95].

Not surprisingly, some cellular proteins seem to use the early endosome to TGN pathway, one of them is TGN38/46, a protein that resides in the TGN but cycles from the cell surface to early endosomes to TGN [96,97]. No known function has been associated to TGN38/46, however, it is commonly used as a marker for TGN. Other proteins such as GPP130 and GP73 reside in the cis GA, but upon treatment of cells with agents that increase the internal pH of GA, and endosomes, these two proteins relocalize to early endosomes, and when GPP130 is overexpressed, part of it redistributes to early endosomes [98-100]. Moreover GPP130 is sialylated and phosphorylated, two post-translational modifications that occur in later GA

compartments. These observations suggest cycling of these two proteins from cis GA to plasma membrane, endosomes, TGN, and cis GA. Recent kinetic studies [101] have shown that the accumulation of GPP130 in early endosomes in response to pH elevators is due to a 3-4 fold decrease in the rate of endosome to GA transport, resulting in increased time of stay of GPP130 in endosomes. Similarly, transport of SLT B subunit (SLTB) in the presence of pH elevator agents slows down the rate of exit of SLTB from early endosomes to TGN, which results in accumulation of SLTB in early endosomes, suggesting that SLTB and GPP130 may either share the same transport system out of endosomes to TGN, or that GPP130 is necessary for transport of proteins form endosomes to the GA [100,101]. Inhibition of GPP130 expression by siRNA results in blocked exit of SLTB from endosomes to TGN, and accumulation of cellular proteins that cycle via this route (GP73 and TGN46) in endosomes, further implying that GPP130 may actually have a functional role in the traffic from endosomes to the GA [100]. These examples establish a major non-degradative/non-lysosomal pathway for drug delivery within cells.

3. NON-ENDOCYTIC/LYSOSOMAL STRATEGIES TO DRUG-TARGETED CELL KILLING

3.1 Killing at the cell surface

Therapeutic and diagnostic antibodies have proliferated in the past years, with several monoclonal antibodies on the market and many on clinical trials [102]. The clinical and commercial success of therapeutic monoclonal antibodies have been possible due to the establishment of monoclonal antibody and recombinant antibody technology, the humanization of murine antibodies, and the capacity to produce large amounts of antibodies through improved bacterial and mammalian expression systems [102,103]. There is a growing list of FDA approved therapeutic antibodies, some examples include, Herceptin® (Trastuzumab), a humanized IgG1 directed against the HER2 receptor (growth factor receptor) and used in the treatment of breast cancer expressing the HER2 receptor; Zenapax® (Daclizimab) humanized anti-CD25 IgG1 used to treat Hodgkin's lymphoma; and Avasatin® (Bevacizumab), humanized anti-vascular endothelial growth factor (VEGF) IgG1, targets the angiogenesis pathway and is used to treat several cancers (colon, ovarian, lung, renal, breast) [102]. Most of these therapeutic antibodies act through antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cell-mediated cytotoxicity (CDCC) directly at the cell surface [102]. Conjugation of these antibodies to radioisotopes, fluorescent molecules, toxins and other cytotoxic compounds provides alternative uses, such as imaging applications and improved cell killing.

3.2 Direct targeting to the cytosol

Approaches to deliver drugs directly to the cytosol have been proposed based on the use of cell penetrating proteins and peptides derived from HIV-1 trans-activating transcriptional activator (TAT). The cell penetrating capacity of these proteins is conferred by a stretch of about 20 amino acids rich in basic residues (lysine and arginine) [1,104]. Presumably, the TAT peptides can enter the cytosol directly from the cell surface, through binding to heparin sulfate proteoglycans, followed by macropinocytosis from plasma membrane lipid rich domains [104], and slow leakage into the cytosol from the macropinosomes. However, the exact mechanism of internalization remains to be elucidated since other studies have shown TAT internalization through other endocytic mechanisms, such as clathrin-dependent, and clathrin-independent/caveolin-dependent pathways. Brooks et al. have suggested that TAT is an opportunistic peptide, since TAT is positively charged, it binds negatively charged lipids and proteins at the cell surface [105]. Therefore, TAT may be internalized along with any of the cell's normal endocytic processes occurring at the plasma membrane. TAT peptides have been used to deliver DNA, antisense oligonucleotides, peptide nucleic acid (PNA), proteins,

antigens, and even 40 nm iron particles into cells [1,104-106]. TAT peptides may be a promising alternative to the use of viral vector delivery systems for gene therapy.

3.3 Liposomal carriers

Small hydrophilic molecules like folate have also been used as targeting ligands [107]. Folate is an essential water-soluble vitamin of the B-complex that participates as a coenzyme in several important biosynthetic pathways, among others the *de novo* biosynthesis of purines and biosynthesis of thymine from uridine. Folate is taken up at the cell surface by the folate receptor through receptor-mediated endocytosis and subsequently released into the cytosol. Although some studies indicate that folate is internalized by the folate receptor via caveolae, a clathrin-independent mechanism, the exact endocytic mechanism is not clear yet [24,107-110]. Several drug delivery systems have been designed using folate as the targeting molecule based on the fact that the folate receptor is overexpressed in several types of cancer, folate is easy to conjugate to other molecules, and commercially available in large pure quantities. One interesting example is the use of folate-receptor targeted liposomes to deliver drugs like doxorubicin [111]. Folate is covalently attached to a lipid anchor (disteaorylphosphatidylethanolamine or cholesterol) and liposomes are prepared incorporating the folate-derived lipids. A portion of the folate-derived lipids assembles into the outside lipid layer of the liposome, therefore the folate molecule is presented at the surface of the liposome while the drug is carried in the liposome hydrophilic interior. The liposomes may contain pH-sensitive lipids to enhance endocytic drug release (see below). The whole liposome complex is internalized by receptor-mediated endocytosis mediated by the folate receptor endocytic pathway.

4. PRACTICAL STRATEGIES TO USE THE CLASSICAL ENDOCYTIC/ LYSOSOMAL PATHWAY TO THERAPEUTIC ADVANTAGE

4.1 Early escape

Several delivery systems have been designed to escape lysosome degradation, one example is liposomes containing pH-responsive polyanionic polymers and lipids such as dioleoylphosphatidylethanolamine (DOPE). At the lower pH of the endosomes, the polyanionic polymers and DOPE change conformation, disrupting the endosomal membrane and causing translocation of its contents into the cytosol [107,112,113]. Similarly, some bacterial toxins, such as diphtheria toxin (DT), have the ability to unfold in the low endosomal pH environment, insert in the membrane and translocate into the cytosol where they exert toxicity [114-116]. In fact, various drug systems have been based on the ability of DT to transfer from endosomes into the cytosol in a pH-dependent manner. For instance the drug Denileukin difitox or Ontak is composed of a truncated form of DT fused with interleukin-2 (IL-2) and it is used in the treatment of several hematologic malignancies [117].

4.2 Exploitation of the degradative properties of the lysosome

Currently, there are two FDA approved murine anti-CD20 antibody-radionuclide conjugates, ibritumomab tuixetan (^{90}Y-Zevalin) and tositumomab (^{131}I-Bexxar) for B cell lymphomas; and a humanized anti-CD33 antibody-cytotoxin conjugate, gemtuzumab ozogamicin (calicheamicin, Mylotarg®) for leukemia [102,118-122]. The cytotoxin in gemtuzumab ozogamicin is N-acetyl-?-calicheamicin dimethylhydrazine, a member of the enediyne antitumor antibiotic family and a highly toxic antibiotic. These immunoconjugates act intracellularly and enter the cell through the endocytic/lysosomal pathway, the antibody is degraded by proteases in the lysosomes and radionuclides or cytotoxins are released into the cytosol. In the case of the cytotoxin immunoconjugates, the linkage between the antibody and the cytotoxin is generally designed for optimal release by taking advantage of pH and enzymes

present in the lysosomes [121]. For example, acid-labile linkers, amino acid linkers recognized by lysosomal proteases, or disulfide bonds, enzymatically reduced in endosomes/lysosomes have been used [102,121,123,124].

The synthetic polymer-drug conjugate (PK2, FCE28069) that entered phase I/II clinical trials for the treatment of primary liver cancer, is based on the [N-(2-hydroxypropyl) methacrylamide] (HPMA) copolymers and the use of a lysosome cleaved linkage [125]. The HPMA core carries the drug, doxorubicin and the targeting ligand, galactosamine, which binds the hepatic asialoglycoprotein receptor. PK2 is thus internalized via the endocytic/lysosomal pathway, is released in the cytosol and accumulates in the nucleus, the site of action. Doxorubicin is linked to HPMA through a tetrapeptide sensitive to cathepsin, a lysosomal protease, enabling a stable linkage during drug transit but optimal intracellular release of active drug at the site of action [125,126]. Phase I evaluation of PK2 demonstrated specific delivery of doxorubicin to hepatocellular tumors and accumulation of PK2 in the tumors was significantly above background.

5. INITIAL STRATEGIES TO USE LYSOSOMAL BYPASS DELIVERY ROUTES

So far, all the drug delivery systems examples presented, utilize receptor-mediated endocytosis and transfer into the cytosol from either early endosomes by pH-mediated mechanisms, or transfer into cytosol from lysosomes using normal cell mechanisms (except, perhaps, for the TAT peptide and the folate/folate receptor entry pathways which are undetermined yet). Nevertheless, as described in previous sections, there are other endocytic pathways in which arrival to the cytosol is accomplished by a longer route, through retrograde membrane traffic from endosomes to the trans-Golgi network (TGN), Golgi apparatus (GA), endoplasmic reticulum (ER), and finally cytosol. The caveosome is also a potential route. In this section we will focus our discussion on ST/SLT and the initial strategies used to deliver drugs to the cytosol by using lysosomal bypass pathways. A summary of these strategies is presented in Table 1.

5.1 Shiga toxins and Shiga-like toxins

The Shiga toxin-group of toxins are produced by *Shigella dysenteriae* type 1 and by some *Escherichia coli* strains [127-130]. These toxins cause severe illnesses, such as hemolytic colitis and hemolytic uremic syndrome. The protein structure of Shiga toxin (ST) consists of two subunits, termed A and B. A single subunit A, 32 kD, associates non-covalently with a pentamer of five identical B subunits monomers, 8 kD in size each, to give the holotoxin (see Figure 2). The *E. coli* related Shiga toxins possess similar structure and biological activity as ST, and hence have been designated as Shiga-like toxins (SLT) [130,131]. Within the SLTs, two types of toxins exist, SLT1 and SLT2. SLT1 cross-reacts with polyclonal antibodies to ST as they both share identical B subunits, and the A subunits differ by merely one amino acid [129-131]. SLT2 has the same binding specificity and biological activity as ST/SLT1 but it is immunologically distinct from ST and SLT1 [131]. The subunit A of ST and SLTs is an *N*-glycosidase that cleaves a unique adenine residue in the 28S rRNA of the 60S ribosomal subunit causing inactivation of protein synthesis that eventually leads to cell death. The subunit B of ST and SLTs (STB and SLTB) binds to the cell surface glycosphingolipid, globotriaosylceramide or Gb_3 (Galactoseα1-4Galactoseα1-4Glucose-Ceramide) promoting receptor-mediated internalization of the holotoxin by the various mechanisms described above. STB and SLTB exhibit high affinity for Gb_3, with a dissociation constant (K_d) in the range of 10^{-17} to 10^{-12} M [132], and about 3 binding sites per B subunit monomer [133]. ST/SLT is synthesized as a non-toxic proprotein, that is activated shortly after endocytosis by proteolytic cleavage of the A subunit by furin in early endosomes [128]. Cleavage of the A subunit by furin produces two fragments (A1 and A2) that are held together by an internal disulfide bond (see Figure 2). Once ST/SLT reaches the ER, the enzyme protein disulfide isomerase releases A1 from the rest of the molecule (A2 and subunit B remain associated) (see Figure 2). A1,

which retains the toxic enzymatic activity, translocates then to the cytosol from the ER, allegedly using the cellular ERAD machinery [128], where it modifies 28S rRNA, subsequently inhibiting protein synthesis and ultimately causing cell death.

5.2 Presentation of major histocompatibility complex class I (MHC-I) antigens in dendritic cells

Due to the unique trafficking pathway of ST and SLT, and the expression of Gb_3 in antigen presenting cells (APC) such as dendritic cells and some B cells [134,135], ST/SLT has been sought as a vector to deliver antigens to the MHC class I pathway of APC. In the classical MHC-I antigen presentation pathway, endogenously synthesized defective cellular proteins and viral proteins are degraded in the cytosol by the proteasome and then loaded into MHC-I molecules in the ER [136]. The antigenic peptides generated in the cytosol by the proteasome are translocated into the ER by specialized ATP-dependent ABC-type transmembrane transporters in the ER membrane associated with antigen processing and presentation (TAP). Once in the ER, antigenic peptides are loaded into newly synthesized MHC-I molecules, thus forming MHC-I/peptide complexes that are delivered to the plasma membrane via the secretory pathway. On the other hand, exogenous antigens enter the endocytic pathway, eventually reaching the late endosomal/lysosomal compartements where they are fragmented into peptides by resident proteases, loaded on MHC-II molecules, and transported to the plasma membrane via the same endosomal system. Even though there is a clear distinction between the presentation of endogenous antigens by MHC-I molecules and exogenous antigens by MHC-II molecules, exogenous antigens can be presented by MHC-I molecules, as well as intracellular antigens can be presented by MHC-II molecules. Some APC, for example dendritic cells, present peptides derived from exogenous antigens in both MHC-II and MHC-I molecules in a process called cross-presentation [136,137]. Exogenous antigens can be loaded on MHC-I molecules following endocytosis. However, how the antigens enter the cytosol from late endosomes/lysosomes is still uncertain. It has been suggested that the antigens leak into the cytosol either by a transitory rupture of the late endosomal/lysosomal membrane or by as yet uncharacterized specific transmembrane channels in the endosomes/lysosomes. Another possibility is that internalized exogenous antigens may use the retrograde pathway from endosomes to the GA and then to the ER as ST/SLT, or they can move directly from endosomes to ER as SV40 virus does, and subsequently may egress from the ER lumen into the cytosol, by making use of the ERAD machinery. Alternatively, there is evidence indicating that exogenously derived peptide antigens may be loaded on MHC-I molecules (possibly recycled MHC-I molecules from the plasma membrane) in the endocytic pathway, in a TAP-independent manner, and therefore do not require transport to the cytosol.

APC bearing MHC-I/peptide complexes are recognized by $CD8^+$ cytotoxic T lymphocytes (CTL), initiating a primary immune response that leads to maturation of CTL, recognition by mature CTL of the MHC-I-presented antigens in diseased cells which may either be virally infected, or sustain genetic abnormalities as for example cancer cells, and subsequent destruction of the diseased cells by activated CTL. Similarly, APC presenting MHC-II/peptide complexes are recognized by $CD4^+$ helper T lymphocytes (T_H1), leading to activation of T_H1, which in turn secrete the cytokine, IL-2. IL-2 promotes growth, differentiation and activation of CTL. Some APC present exogenous antigens in MHC-I and MHC-II molecules, therefore they are able to produce activation of CTL and induce cell-mediated and humoral immune responses necessary to eliminate virus infected and cancer cells. Several strategies have been designed to artificially stimulate APC to present exogenous antigens in the MHC-I pathway to induce immune protection towards viral infections and tumors. One innovative approach is the use of STB as a vector to deliver exogenous antigens to the MHC-I pathway of APC, devised by the group of Johannes and Tartour [134,137-141]. In initial studies, Lee et al. [138] showed that recombinant fusion proteins composed of STB and a model tumor

antigen, Mage 1, were internalized by peripheral blood mononuclear lymphocytes (PBML) and presented in the MHC-I pathway, independently of carrying an active or inactive ER retrieval signal (KDEL and KDELGL), to Mage 1-specific CTL. Epstein-Barr virus (EBV) transformed-B cells and dendritic cells pulsed with STB-Mage 1 fusion protein were also able to present this antigen in an MHC-1-restricted pathway to Mage 1-specific CTL. T cells, which do not express Gb_3, when pulsed with STB-Mage 1, could not present STB-Mage 1-derived antigens. Treatment of EBV-B cells with BFA, which negatively affects STB transport to the ER and transport of MHC-I/peptide complexes to the plasma membrane, inhibited presentation of STB-Mage 1 in the MHC-I pathway and failed to activate Mage 1-specific CTL, indicating that internalization of STB-Mage 1 is necessary for MHC-I antigen presentation. Also, immunofluorescent analysis showed that STB-Mage 1 accumulated in ER and GA of EBV-B cells, and did not colocalize with compartments labeled with lysosomal markers. These initial findings revealed that indeed STB could be used as a non-living, non-toxic vaccine vector. In related studies, Haicheur et al. [134] reported that STB fused to a tumor peptide derived from mouse mastocytoma P815 induces specific CTL in mice without the need of adjuvant. They also show that in vitro, STB fused to other exogenous antigenic peptides, targets them to the MHC-I pathway of mouse dendritic cells. The process is dependent on internalization via receptor-mediated endocytosis since antibodies against STB, or inhibition of Gb_3 synthesis prevented antigen presentation. STB delivery of exogenous antigens to the MHC-I pathway in mouse dendritic cells is blocked by treatment with BFA, and with lactacystin, a specific proteasome inhibitor, indicating that STB targets exogenous antigens to the classic MHC-I presentation pathway. Also, mouse dendritic cells deficient in TAP failed to present STB-delivered antigens confirming the latter results. Interestingly, in another study, Haicheur et al. [139] found that chemically coupled STB to ovalbumin (OVA) (STB-OVA) delivered OVA-derived peptides into both MHC-I and MHC-II antigen presentation pathways in mouse dendritic cells. When the STB trafficking pathway in mouse dendritic cells was investigated, it was found that not all internalized STB followed the retrograde transport pathway to the ER, there was a portion of STB that colocalized with late endosomal/lysosomal compartments, explaining how STB targets exogenous antigens to the MHC-II pathway. However, these results do not establish the mechanisms by which STB delivers exogenous antigens to the MHC-I pathway. A fraction of STB-OVA that follows retrograde transit to ER may translocate to the cytosol from this site, nevertheless, there is no evidence that STB itself transfers to the cytosol from ER. When ST holotoxin reaches the ER, only the catalytic ST A subunit translocates to the cytosol. There is also the possibility that STB in mouse dentritic cells may enter the cytosol by translocating from late endosomes/lysosomes. An earlier study demonstrated that in human monocyte dendritic cells, STB does not use the retrograde route to ER as it is the case in HeLa cells, but STB accumulates in late endosome/lysosomes [66]. It seems that localization of STB in late endosomes/lysosomes of human dendritic cells was related to the fact that Gb_3 is not organized in detergent-resistant microdomains (lipid rafts) in the plasma membrane of these cells, as compared to HeLa cells [66]. Mice vaccinated with STB-OVA produced specific anti-OVA CTL response, as well as anti-OVA humoral immune response without the use of adjuvants [139,140]. Splenocytes and T_H1 cells from SLTB-OVA-immunized mice secreted higher levels of gamma interferon (IFN-γ) and anti-OVA IgG2a-type antibodies as compared to mice immunized with OVA only [139]. In addition, OVA-specific $CD8^+$ T cells (CTL) were detected *ex vivo* in mice immunized with STB-OVA, these cells were long lasting as they could be detected up to 91 days after the last vaccination [140]. Mice depleted of dendritic cells and immunized with STB-OVA failed to exhibit OVA-specific CTL immune response, indicating that dendritic cells are required in this process. Also, mice immunized with STB coupled to a tumor antigen (E7) protected the mice against challenge with tumor cells expressing the E7 antigen [140]. The fact that Gb_3 is expressed in various mice and human dendritic cells, and that STB when used as a vector to deliver exogenous antigens, *in vivo*, to mice resulted in the stimulation of strong and durable CTL and humoral

immune responses and tumor protection, all point to the potential of STB as a non-live, non-toxic vaccine delivery system for therapy of cancer and infectious diseases.

5.3 Targeted cancer therapy and imaging

The expression and metabolism of cell-surface glycolipids and glycoproteins is altered during oncogenic transformation, and numerous tumor-associated antigens correspond, in fact, to glycosyl structures [141,142]. Even though the molecular basis for the glycosylation changes observed in cancer cells is largely unknown, recent studies indicate that aberrant glycosylation may have a role in tumor invasion and metastasis [141,142]. The expression of globotriaosylceramide or Gb_3 (also known as CD77 and P^k antigen), the receptor for ST/SLT, is enhanced in various cancers relative to expression in the corresponding normal tissue. Gb_3 expression is significantly increased in ovarian carcinoma [143-145], lymphomas [135,146], myelomas [135], breast cancer cells [135], astrocytoma cells [147], malignant meningiomas [148], colon cancer [149], and testicular cancer [150,151]. Furthermore, Gb_3 is expressed in metastatic tumors originating from ovarian [144,145], breast [135] and colon carcinomas [149]. On the other hand, Gb_3 is expressed in several normal tissues throughout the body [152-154], including kidney epithelium, and endothelial cells, in addition to being found in human milk [155]. Gb_3 is also expressed in subsets of dendritic cells and germinal center B-lymphocytes characterized as CD77 positive [134,135,156,157]. Gb_3 is strongly expressed in red blood cell membranes of P^k blood type individuals (0.01% of the population), while only traces are found in red blood cell membranes of most of the population [158].

The specific binding of ST/SLT to Gb_3, coupled with the increased levels of Gb_3 found in particular cancers, have led to the development of strategies that utilize ST/SLT to target these cancerous tissues. In initial studies conducted by Lingwood's group, SLT1, the complete holotoxin, has been used to kill several human ovarian-derived tumor cells, including multi-drug resistant variants [144]. They found that SLT1 was effective in killing ovarian tumor cells with LD_{50}s ranging from 0.001 to100 ng/ml of SLT1 depending on the level of Gb_3 expression of individual cell lines. Interestingly, they found that the multidrug-resistant ovarian tumor cell variants, which contained higher Gb_3 levels, were more sensitive to SLT1 than the drug-sensitive parental cell line that expressed less Gb_3. Additionally, SLT1 prevented growth of metastatic tumors to the lung in a murine metastatic fibrosarcoma model, and SLT1 effect was abrogated in mice immunized with SLTB1. In this study, there were several interesting findings, for instance, surgically removed primary ovarian tumors contained increased levels of Gb_3 relative to normal ovaries. Ovarian metastases showed considerably high Gb_3 levels. And Gb_3 was present in both the tumor-like gland tissue, and the tumor vasculature [144], suggesting a potential role for SLT1 as an antiangiogenic agent [159]. Further studies by Arab et al. [145] on the expression of Gb_3 in ovarian hyperplasias demonstrated that Gb3 was present in both benign and malignant tumors, and the highest Gb_3 content was observed in secondary ovarian metastases and turmors refractory to chemotherapy. Also, they reported high Gb_3 expression in undifferentiated neoplastic ovarian tissue, not only in the tumor mass, but also in blood vessels adjacent and within the tumor. Other studies have shown that astrocytoma cells, derived from primary human brain tumors, are sensitive to SLT1 cytotoxicity at doses around 50 ng/ml [147]. In astrocytoma cells, SLT1 caused cell death by apoptosis. Also, STB binding and internalization alone was able to produce apoptosis in astrocytoma cells [147]. Other brain tumors such as malignant meningiomas are sensitive to killing with SLT1, both *in vitro* and *in vivo* [148].

Other applications of SLT in cancer therapy are illustrated by the work of Gariepy and colleagues, who have applied SLT-1 in the *ex-vivo* purging of malignant cells (expressing Gb_3) from autologous stem cell grafts of breast cancer, lymphoma and myeloma patients [135]. LaCasse et al. [135] reported expression of Gb_3 in 13 out of 18 breast cancer cell lines

tested, including cell lines derived from breast cancer metastasis. Sensitivity to the toxin was correlated, although not linearly, with level of Gb_3 expression in the breast cancer cell lines, with LD_{50}s ranging from as low as 0.01 ng/ml to 40 ng/ml. Also, 8 out of 10 primary breast cancer biopsies screened showed Gb_3 expression. In the 134 tumor samples obtained from hematological cancers, Gb_3 was expressed in several types of lymphomas and myelomas. *Ex-vivo* SLT-1 treatment of various lymphoma and myeloma samples resulted in depletion of malignant B cells by 3-28 fold, whereas normal hematopoietic progenitor cells, which are Gb_3 negative, were unaffected by SLT1 and remained functionally intact. These results clearly indicate that SLT1 may be useful as an *ex-vivo* purging agent to eliminate malignant cells from autologous stem cell grafts.

Kovbasnjuk et al. [149] have studied the expression of Gb_3 in colon cancer and they report that Gb_3 is significantly expressed in primary lesions of metastatic colon cancer and in liver metastases while Gb_3 is absent in normal colonic epithelium. Furthermore, in human colon cell lines, they identified a subpopulation of cells expressing high levels of Gb_3 that at the same time displayed invasive characteristics. Normal polarized epithelial cells devoid of endogenous Gb_3, were transfected with Gb_3 synthase, which resulted in Gb3 expression and induction of cell invasiveness. Similarly, inhibition of Gb_3 synthesis in colon cancer epithelial cells by treatment with siRNA blocked cell invasiveness, suggesting that subpopulations of Gb_3-expressing cells may be involved in the process of tumor cell invasion and metastasis in colon cancer. In addition, treatment of human colon cancer cells with STB, selectively killed Gb_3-positive cells by apoptosis. STB treatment of nude mice bearing colon cancer cell grafts inhibited tumor growth while tumor grafts in STB-untreated nude mice continued to grow.

In a recent study by Storrie and his group [160], SLTB was tested as a carrier to deliver the photosensitizer Chlorin e6 (Ce6) to cells expressing Gb_3 (Vero cells) as a multilevel approach to achieve selective cell killing. SLTB targeting provides one level of selection while confined activation of the photosensitizer by local illumination, provides a second. Ce6 was chosen both for its phototoxic properties and its potential for covalent conjugation to SLTB. However, as other molecules structurally related to porphyrins, Ce6 tends to self-aggregate in aqueous solutions due to its hydrophobicity and planar structure, and can associate non-specifically to hydrophobic regions of proteins. As a result when Ce6 was covalently coupled to SLTB, the Ce6-SLTB conjugate contained 10% non-covalently associated Ce6, even after several cleanup steps, including affinity chromatography. Ce6-SLTB provided a mixed delivery system in Vero cells in which Ce6 accumulated in the GA and ER, reflecting typical intracellular distribution of SLTB, and in mitochondria and other cellular membranes representing the distribution of free Ce6. Importantly, the Ce6-SLTB conjugate enhanced delivery of Ce6 to Vero cells by one order of magnitude as compared to free Ce6, and delivery was receptor-dependent as demonstrated by competitive inhibition studies, indicating that Ce6-SLTB behaved as a vehicle to effectively deliver Ce6 to Vero cells. Furthermore, improved intracellular Ce6 delivery by Ce6-SLTB paralleled Ce6-SLTB-mediated cell phototoxicity, as Ce6-SLTB was 12-fold more photodynamically toxic than free Ce6, displaying an LD_{50} of about 0.1 μM Ce6-SLTB expressed as Ce6 content (≈ 4 μg/ml SLTB). Being Ce6 a fluorescent molecule and based on average measured Ce6 fluorescence per cell, cell killing appeared to be proportional to Ce6 fluorescence accumulation in cells, irrespective of whether Ce6 was delivered via SLTB or as free Ce6. Therefore, it was concluded that there was little enhancement or detriment to cell killing from having Ce6 delivered to multiple subcellular sites as is the case with Ce6-SLTB versus free Ce6 delivery. Much of the cell killing in either case was presumably due to delivery to mitochondria. Whether photosensitizer delivery to strictly GA and ER can produce similar levels of cell killing remains to be elucidated and it will require further studies with more hydrophilic and less planar photosensitizers, with reduced tendency to be absorbed to proteins and to self-aggregate. The ER as a major calcium store within cells is a potential source of apoptotic cell killing. Recent evidence has implicated the GA in apoptosis [161]. Despite the

uncertainty if delivery of Ce6 by Ce6-SLTB to multiple subcellular sites is preferable to a single site, this study shows that Ce6-SLTB delivery of Ce6 increased cell-killing efficacy relative to free Ce6, and highlights a prospective function for SLTB (or STB) as a vehicle to deliver not only photosensitizers but other therapeutic drugs to target cells.

The potential role of STB/SLTB in the targeted visualization of tumors logically follows and complements the use of STB/SLTB in the targeted therapy of tumors. As we have mentioned, the STB/SLTB receptor is expressed in various cancers, including metastatic tumors. The detection and localization of tumors is essential to cancer treatment. Several non-invasive techniques to visualize tumors have been developed and are currently in use, comprising X-ray, computed tomography (CT) scan, nuclear scan, positron emission tomography (PET) scan, magnetic resonance imaging (MRI), confocal laser endoscopy and ultrasound. In general, all these techniques share in common a limited sensitivity to detect small tumors. Detection of incipient tumors is key to patient prognosis and survival. Therefore, visualization techniques with high sensitivity and the ability to discriminate between healthy and cancerous tissues would constitute a significant advantage in early cancer detection and treatment. The role of STB/SLTB in tumor imaging has been proposed earlier [162] and certainly, visualizing agents may be chemically attached to STB/SLTB, like for example, radioactive isotopes (e.g. technetium-99, iodine-125, thallium-201, fluor-18), paramagnetic contrast agents (e.g. gadolinium, iron oxides), and fluorescent dyes (e.g. Cy3, fluorescein). Moreover, STB/SLTB can be genetically altered to include amino acids with side chains suitable for covalent linkage to other molecules [139], as well as to make chimeric proteins containing for instance GFP. A new study conducted by Johannes and colleagues [163] explores the use of STB in the targeted imaging of digestive tumors in transgenic mice carrying oncogenes associated to spontaneous digestive tumorigenesis. Mouse spontaneous intestinal adenocarcinomas were found to express Gb_3 and were visualized *in situ* by confocal laser endoscopy in conjunction with oral administration of fluorophore-labelled STB to transgenic mouse. They found that fluorophore-labelled STB strongly accumulated in the tumor lesions of affected mouse, and this accumulation was verified to be STB-dependent by immunocytochemistry. Control experiments with wild-type mouse demonstrated that only isolated single cells, corresponding to enteroendocrine cells, were visible in the intestinal epithelium. In other experiments, fluor-18-labelled STB was delivered intravenously to mice before performing whole body PET imaging. STB-targeted PET imaging showed that the majority of radioactivity accumulated in the urinary tract of both transgenic and wild-type mice. Lower levels of radioactivity were also observed in the spleen, lungs and liver. In wild-type mice no radioactivity was associated with the intestinal tract but in a transgenic mouse two areas of the digestive tract showed strong labeling that were later confirmed to be Gb_3-positive colon adenocarcinoma tumors. Analysis of STB intracellular transport in primary cell cultures established from mouse colon adenocarcinoma tumors, demonstrated that in these cells after internalization, STB followed the retrograde transport pathway to GA and ER, indicating that tumor visualization was indeed provided by specific and stable association of STB with the tumor cells. These results are surely promising as they open a broad window of applications for STB/SLTB, as for example, aid in detection of metastases, selection of tumor sites to perform biopsies, and mapping of tumor areas to be surgically removed. Nevertheless, the short and long term side effects of STB/SLTB to humans and animals are practically unknown and require in depth investigation as part of the process of identifying STB/SLTB as a therapeutic and diagnostic tool.

6. Conclusions and perspectives for future work

We have presented here the multiplicity of pathways for delivery of drugs to the cell interior. In doing so, we have highlighted retrograde trafficking pathways between endosomes and the endoplasmic reticulum. The Golgi apparatus is frequently central to these pathways. The existence of pathways for drug or antigen delivery to the endoplasmic reticulum and Golgi

apparatus has been to a large extent an outcome of research on the trafficking of bacterial or plant toxins such as Shiga toxin within the cell. The targeting properties of these toxins reside in their B subunit. For Shiga toxin, the B subunit binds to the glycolipid Gb_3. Because the B subunits can be produced as standalone polypeptides through recombinant DNA techniques, one can work with the B subunit as a non-toxic carrier protein for drug and antigen delivery and for imaging. Potential applications to drug delivery and imaging are numerous and we have highlighted some of these.

Clearly, there is potential today to deliver reagents selectively to numerous different subcellular compartments. However, whether one compartment or another should be preferred remains an open question. The answer depends to a large extent on purpose. If the purpose is antigen delivery and a MHC type I response, then delivery to the endoplasmic reticulum is the obvious goal. If the goal is invoked cell killing, for example, through a photosensitizer, then the answer is less obvious. The Golgi apparatus and endoplasmic reticulum are less well developed as apoptotic sites than the mitochondria. However, recent work suggests that damage to the Golgi apparatus can produce apoptotic signals and hence may well be an appropriate site for the delivery of cytotoxic reagents [161].

In conclusion, cell biology has revealed a new range of intracellular delivery sites that may well have unique advantages. These provide new opportunities for treatments. Their exploitation will require a repeated trial and error interdisciplinary research process. This process is referred to as translational research, the process of going from laboratory bench to bedside. The bridging of this gap will be an important challenge for the future. Our hope is that in these possibilities will also be seen by others as an intriguing challenge.

Abbreviations

APC, antigen presenting cells; CT, Cholera toxin; CTL, cytotoxic T lymphocytes; DT, Diphtheria toxin; HLE, *E. coli* heat labile toxin; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; GA, Golgi apparatus; Gb_3, globotriaosylceramide; GFP, green fluorescent protein; GM1, ganglioside GM1; IL-2, interleukin-2; M6PR, mannose-6-phosphate receptor; MHC, major histocompatibility complex; PE, *Pseudomonas* exotoxin; ST, Shiga toxin; SLT, Shiga-like toxin; STB/SLTB, ST/SLT subunit B; SV40, simian virus 40; TAT, HIV-1 trans-activating transcriptional activator; T_H1, helper T lymphocytes; TGN, trans-Golgi network.

References

[1]. Torchilin VP. Recent approaches to intracellular delivery of drugs and DNA and organelle targeting. Annu. Rev. Biomed. Eng 2006;8:43–75.

[2]. Johannes L, Lamaze C. Clathrin-dependent or not: is it still the question? Traffic 2002;3:443–451. [PubMed: 12047552]

[3]. Mousavi SA, Malerod L, Berg T, Kjeken R. Clathrin-dependent endocytosis. Biochem J 2004;377:1–16. [PubMed: 14505490]

[4]. Mellman I, Warren G. The road taken: past and future foundations of membrane traffic. Cell 2000;100:99–112. [PubMed: 10647935]

[5]. Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T, Bonifacino JS. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science 1995;269:1872–1875. [PubMed: 7569928]

[6]. Simonsen A, Bremnes B, Nordeng TW, Bakke O. The leucine-based motif DDQxxLI is recognized both for internalization and basolateral sorting of invariant chain in MDCK cells. Eur. J. Cell Biol 1998;76:25–32. [PubMed: 9650780]

[7]. Ford MG, Pearse BM, Higgins MK, Vallis Y, Owen DJ, Gibson A, Hopkins CR, Evans PR, MacMahon HT. Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. Science 2001;291:1051–1055. [PubMed: 11161218]

[8]. Takei K, Haucke V. Clathrin-mediated endocytosis: membrane factors pull the trigger. Trends Cell Biol 2001;11:385–391. [PubMed: 11514193]

[9]. Jin AJ, Nossal R. Topological mechanisms involved in the formation of clathrin-coated vesicles. Biophys. J 1993;65:1523–1537. [PubMed: 8274646]

[10]. Schmidt AA. Membrane transport: the making of a vesicle. Nature 2002;419:347–349. [PubMed: 12353016]

[11]. Kozlov MM. Fission of biological membranes: interplay between dynamin and lipids. Traffic 2001;2:51–65. [PubMed: 11208168]

[12]. Lee E, De Camilli P. Dynamin at actin tails. Proc. Natl. Acad. Sci. U.S.A 2002;96:161–166. [PubMed: 11782545]

[13]. Merrifield CJ, Feldman ME, Wan L, Almers W. Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. Nat. Cell Biol 2002;4:691–698. [PubMed: 12198492]

[14]. Subtil A, Gaidarov I, Kobylarz K, Lampson MA, Keen JH, McGraw TE. Acute cholesterol depletion inhibits clathrin-coated pit budding. Proc. Natl. Acad. Sci. U.S.A 1999;96:6775–6780. [PubMed: 10359788]

[15]. Rodal SK, Skeretting G, Garred O, Vilhardt F, van Deurs B, Sandvig K. Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. Mol. Biol. Cell 1999;10:961–974. [PubMed: 10198050]

[16]. Lamaze, C.; Johannes, L. Intracellular trafficking of bacterial and plant protein toxins. In: Alouf, JE.; Popoff, MR., editors. The Comprehensive Sourcebook of Bacterial Protein Toxins. third edition. Academic Press; Paris: 2006. p. 135-153.

[17]. Laude AJ, Prior IA. Plasma membrane microdomains: organization, function and trafficking. Mol. Memb. Biol 2004;21:193–205.

[18]. Simons K, Ikonen E. Functional rafts in cell membranes. Nature 1997;387:569–572. [PubMed: 9177342]

[19]. Foster LJ, De Hoog CL, Mann M. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. Proc. Natl. Acad. Sci. U.S.A 2003;100:5813–5818. [PubMed: 12724530]

[20]. Simons K, Toomre D. Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol 2000;1:31–39. [PubMed: 11413487]

[21]. Palade GE. Fine structure of blood capillaries. J. Appl. Phys 1953;24:1424.

[22]. Yamada E. The fine structure of the gall bladder epithelium of the mouse. J. Biophys. Biochem. Cytol 1955;1:445–458. [PubMed: 13263332]

[23]. Couet J, Belanger MM, Roussel E, Drolet MC. Cell biology of caveolae and caveolin. Adv. Drug Del. Rev 2001;49:223–235.

[24]. Rothberg KG, Heuser JE, Donzell WC, Ying YS, Genney JR, Anderson RG. Caveolin, a protein component of caveolae membrane coats. Cell 1992;68:673–678. [PubMed: 1739974]

[25]. Harder T, Simons K. Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. Curr. Opin. Cell Biol 1997;9:534–542. [PubMed: 9261060]

[26]. Sharma DK, Brown JC, Choudhury A, Peterson TE, Holicky E, Marks DL, Simari R, Parton RG, Pagano RE. Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol. Mol. Biol. Cell 2004;15:3114–3122. [PubMed: 15107466]

[27]. Shajahan AN, Timblin BK, Sandoval R, Tiruppathi C, Malik AB, Minshall RD. Role of Src-induced dynamin-2 phosphorylation in caveolae-mediated endocytosis in endothelial cells. J. Biol. Chem 2004;279:20392–20400. [PubMed: 15007081]

[28]. Nichols BJ, Lippincott-Schwartz J. Endocytosis without the clathrin coats. Trends Cell Biol 2001;13:406–412. [PubMed: 11567873]

[29]. Nichols B. Caveosomes and endocytosis of lipid rafts. J. Cell Sci 2003;116:4707–4714. [PubMed: 14600257]

[30]. Schlegel A, Lisanti MP. A molecular dissection of caveolin-1 membrane attachment and oligomerization. Two separate regions of the caveolin-1 C-terminal domain mediate membrane binding and oligomer/oligomer interaction *in vivo*. J. Biol. Chem 2000;275:21605–21617. [PubMed: 10801850]

[31]. Thomsen P, Roepstorff K, Stahlhut M, van Deurs B. Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. Mol. Biol. Cell 2002;13:238–250. [PubMed: 11809836]

[32]. Fujimoto T, Miyawaki A, Mikoshiba K. Inositol 1,4,5-trisphosphate receptor-like protein in plasmalemmal caveolae is linked to actin filaments. J. Cell Sci 1995;108:7–15. [PubMed: 7738118]

[33]. Stahlhut M, van Deurs B. Identification of filamin as a novel ligand for caveolin-1: evidence for the organization of caveolin-1-associated membrane domains by the actin cytoskeleton. Mol. Biol. Cell 2000;11:325–337. [PubMed: 10637311]

[34]. Parton RG, Joggerst B, Simons K. Regulated internalization of caveolae. J. Cell Biol 1994;127:1199–1215. [PubMed: 7962085]

[35]. Le PU, Guay G, Altschuler Y, Nabi IR. Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum. J. Biol. Chem 2002;277:3371–3379. [PubMed: 11724808]

[36]. Pelkmans L, Kartenbeck J, Helenius A. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular transport pathway to the ER. Nat. Cell Biol 2001;3:473–483. [PubMed: 11331875]

[37]. Pelkmans L, Puntener D, Helenius A. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. Science 2002;296:535–539. [PubMed: 11964480]

[38]. Damm EM, Pelkmans L, Kartenbeck J, Mezzacasa A, Kurzchalia T, Helenius A. Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. J. Cell Biol 2005;168:477–488. [PubMed: 15668298]

[39]. Torgersen ML, Skretting G, van Deurs B, Sandvig K. Internalization of Cholera toxin by different endocytic mechanisms. J. Cell Sci 2001;114:3737–3747. [PubMed: 11707525]

[40]. Singh RD, Puri V, Valiyaveettil JT, Marks DL, Bittman R, Pagano RE. Selective caveolin-1-dependent endocytosis of glycosphingolipids. Mol. Biol. Cell 2003;14:3254–3265. [PubMed: 12925761]

[41]. Nichols BJ. A distinct class of endosomes mediates clathrin-independent endocytosis to the Golgi complex. Nat. Cell Biol 2002;4:374–378. [PubMed: 11951093]

[42]. Kirkham M, Fujita A, Chadda R, Nixon SJ, Kurzchalia TV, Sharma DK, Pagano RE, Hancock JF, Mayor S, Parton RG. Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. J. Cell Biol 2005;168:465–476. [PubMed: 15668297]

[43]. Massol RH, Larsen JE, Fujinaga Y, Lencer WI, Kirchhausen T. Cholera toxin toxicity does not require functional Arf6- and dynamin-dependent endocytic pathways. Mol. Biol. Cell 2004;15:3631–3641. [PubMed: 15146065]

[44]. Pang H, Le PU, Nabi IR. Ganglioside GM1 levels are a determinant of the extent of caveolae/raft-dependent endocytosis of Cholera toxin to the Golgi apparatus. J. Cell Sci 2004;117:1421–1430. [PubMed: 14996913]

[45]. Parton RG. Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. J. Histochem. Cytochem 1994;42:155–166. [PubMed: 8288861]

[46]. Oh P, McIntosh DP, Schnitzer JE. Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. J. Cell Biol 1998;141:101–114. [PubMed: 9531551]

[47]. Orlandi PA, Fishman PH. Filipin-dependent inhibition of Cholera toxin: evidence for toxin internalization and activation through caveolae like domains. J. Cell Biol 1998;141:905–915. [PubMed: 9585410]

[48]. Tsai B, Gilbert JM, Stehle T, Lencer W, Benjamin TL, Rapoport TA. Gangliosides are receptors for murine polyoma virus and SV40. EMBO J 2003;22:4346–4355. [PubMed: 12941687]

[49]. Lauvrak SU, Torgersen ML, Sandvig K. Efficient endosome-to-Golgi transport of Shiga toxin is dependent on dynamin and clathrin. J. Cell Sci 2004;117:2321–2331. [PubMed: 15126632]

[50]. Iversen TG, Skretting G, van Deurs B, Sandvig K. Formation of clathrin-coated pits with long dynamin-wrapped necks upon inducible expression of antisense clathrin. Proc. Natl. Acad. Sci. USA 2003;100:5175–5180. [PubMed: 12682302]

[51]. Saint-Pol A, Yelamos B, Amessou M, Mills I, Dugast M, Tenza D, Schu P, Antony C, McMahon HT, Lamaze C, Johannes L. Clathrin adaptor epsinR is required for retrograde sorting on early endosomal membranes. Dev. Cell 2004;6:525–538. [PubMed: 15068792]

[52]. Mallard F, Tenza D, Antony C, Salamero J, Goud G, Johannes L. Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of Shiga toxin-B fragment transport. J. Cell Biol 1998;143:973–990. [PubMed: 9817755]

[53]. Cabiaux V. pH-sensitive toxins: interactions with membrane bilayers and application to drug delivery. Ad. Drug Del. Rev 2004;56:987–997.

[54]. Watson P, Spooner RA. Toxin entry and trafficking in mammalian cells. Ad. Drug Del. Rev 2006;58:1581–1596.

[55]. Sandvig K, Garred O, Prydz K, Kozlov JV, Hansen SH, van Deurs B. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature 1992;358:510–512. [PubMed: 1641040]

[56]. Schapiro FB, Lingwood C, Furuya W, Grinstein S. pH-independent retrograde targeting of glycolipids to the Golgi complex. Am. J. Physiol 1998;274:C319–C332. [PubMed: 9486120]

[57]. Dahms NM, Lobel P, Kornfeld S. Mannose 6-phosphate receptors and lysosomal enzyme targeting. J. Biol. Chem 1989;264:12115–12118. [PubMed: 2545698]

[58]. Lombardi D, Soldati T, Riederer MA, Goda Y, Zerial M, Pfeffer SR. Rab9 functions in transport between late endosomes and trans Golgi network. EMBO J 1993;12:677–682. [PubMed: 8440258]

[59]. Sandvig K, Grimmer S, Lauvrak SU, Torgersen ML, Skretting G, van Deurs B, Iversen TG. Pathways followed by Ricin and Shiga toxin into cells. Histochem. Cell Biol 2002;117:131–141. [PubMed: 11935289]

[60]. Iversen TG, Skretting G, LLorente A, Nicoziani P, van Deurs B, Sandvig K. Endosome to Golgi transport of Ricin is independent of clathrin and of the Rab9-Rab11-GTPases. Mol. Biol. Cell 2001;12:2099–2107. [PubMed: 11452006]

[61]. Lauvrak S, Llorente A, Iversen TG, Sandvig K. Selective regulation of the Rab9-independent transport of ricin to the Golgi apparatus by calcium. J. Cell Sci 2002;115:3449–3456. [PubMed: 12154075]

[62]. Wilcke M, Johannes L, Galli T, Mayau V, Goud B, Salamero J. Rab11 regulates the compartmentalization of early endosomes required for efficient transport from early endosomes to the trans-Golgi network. J. Cell Biol 2000;151:1207–1220. [PubMed: 11121436]

[63]. Monier S, Jollivet F, Janoueix-Lerosey I, Johannes L, Goud B. Characterization of novel Rab6-interacting proteins involved in endosome-to-TGN transport. Traffic 2002;3:289–297. [PubMed: 11929610]

[64]. Mallard F, Tang DBL, Galli T, Tenza D, Saint-Pol A, Yue X, Antony C, Hong WJ, Goud B, Johannes L. Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. J. Cell Biol 2002;156:653–664. [PubMed: 11839770]

[65]. Amessou M, Fradagrada A, Falguieres T, Lord JM, Smith DC, Roberts LM, Lamaze C, Johannes L. Syntaxin 16 and syntaxin 5 are required for efficient retrograde transport of several exogenous and endogenous cargo proteins. J. Cell Sci 2007;120:1457–1468. [PubMed: 17389686]

[66]. Falguieres T, Mallard F, Baron C, Hanau D, Lingwood C, Goud B, Salamero J, Johannes L. Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent-resistant membranes. Mol. Biol. Cell 2001;12:2453–2468. [PubMed: 11514628]

[67]. Tai G, Lu L, Wang TL, Tang BL, Goud B, Johannes L, Hong W. Participation of the syntaxin 5/Ykt6/GS28/GS15 SNARE complex in transport from early/recycling endosome to the trans-Golgi network. Mol. Biol. Cell 2004;15:4011–4022. [PubMed: 15215310]

[68]. Storrie B. Maintenance of Golgi apparatus structure in the face of continuous protein recycling to the endoplasmic reticulum: making ends meet. Int. Rev. Cytol 2005;244:69–94. [PubMed: 16157178]

[69]. Marsh BJ, Howell KE. The mammalian Golgi-complex debates. Nature Rev. Mol. Cell Biol 2002;3:789–795. [PubMed: 12360195]

[70]. Farquhar MG, Palade GE. The Golgi apparatus: 100 years of progress and controversy. Trends Cell Biol 1998;8:2–10. [PubMed: 9695800]

[71]. van Vliet C, Thomas EC, Merino-Trigo A, Teasdale RD, Gleeson PA. Intracellular sorting and transport of proteins. Prog. Biophys. Mol. Biol 2003;83:1–45. [PubMed: 12757749]

[72]. Altan-Bonnet N, Sougrat R, Lippincott-Schwartz J. Molecular basis for Golgi maintenance and biogenesis. Curr. Op. Cell Biol 2004;16:363–372.

[73]. Johannes L, Tenza D, Antony C, Goud B. Retrograde transport of KDEL-bearing B-fragment of Shiga Toxin. J. Biol. Chem 1997;272:19554–19561. [PubMed: 9235960]

[74]. Fujinaga Y, Wolf AA, Rodighiero C, Wheeler H, Tsai B, Allen L, Jobling MG, Rapoport T, Holmes RK, Lencer WI. Gangliosides that associate with lipid rafts mediate transport of Cholera and related toxins from the plasma membrane to the endoplasmic reticulum. Mol. Biol. Cell 2003;14:4783–4793. [PubMed: 13679513]

[75]. Hessler JL, Kreitman RJ. An early step in Pseudomonas exotoxin action is removal of the terminal lysine residue, which allows binding to the KDEL receptor. Biochemistry 1997;36:14577–14582. [PubMed: 9398176]

[76]. Smith DC, Spooner RA, Watson PD, Murray JL, Hodge TW, Amessou M, Johannes L, Lord JM, Roberts LM. Internalized Pseudomonas exotoxin A can exploit multiple pathways to reach the endoplasmic reticulum. Traffic 2006;7:379–393. [PubMed: 16536737]

[77]. Lippincott-Schwartz J, Yuan L, Bonifacino JS, Klausner RD. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell 1989;56:801–813. [PubMed: 2647301]

[78]. Sciaky N, Presley J, Smith C, Zaal KJ, Cole N, Moreira JE, Terasaki M, Siggia E, Lippincott-Schwartz J. Golgi tubule traffic and the effects of brefeldin A visualized in living cells. J. Cell Biol 1997;139:1137–1155. [PubMed: 9382862]

[79]. Girod A, Storrie B, Simpson JC, Johannes L, Goud B, Roberts LM, Lord JM, Nilsson T, Pepperkok R. Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum. Nat. Cell Biol 1999;1:423–430. [PubMed: 10559986]

[80]. Ward TH, Polishchuk RS, Caplan S, Hirschberg K, Lippincott-Schwartz J. Maintenance of Golgi structure and function depends on the integrity of ER export. J. Cell Biol 2001;155:557–570. [PubMed: 11706049]

[81]. Puri S, Linstedt AD. Capacity of the Golgi apparatus for biogenesis from the endoplasmic reticulum. Mol. Cell Biol 2003;14:5011–5018.

[82]. Storrie B, White J, Rottger S, Stelzer EH, Suganuma T, Nilsson T. Recycling of Golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides explanation for nocodazole-induced Golgi scattering. J. Cell Biol 1998;143:1505–1521. [PubMed: 9852147]

[83]. Miles S, McManus H, Forsten KF, Storrie B. Evidence that the entire Golgi apparatus cycles in interphase HeLa cells: sensitivity of Golgi matrix proteins to an ER exit block. J. Cell Biol 2001;155:543–555. [PubMed: 11696556]

[84]. Rhee SW, Starr T, Forsten-Williams K, Storrie B. The steady-state distribution of glycosyltransferases between the Golgi apparatus and the endoplasmic reticulum is approximately 90:10. Traffic 2005;6:978–990. [PubMed: 16190979]

[85]. Seemann J, Jokitalo E, Pypaert M, Warren G. Matrix proteins can generate the higher order architecture of the Golgi apparatus. Nature 2000;407:1022–1026. [PubMed: 11069184]

[86]. Stroud WJ, Jiang S, Jack G, Storrie B. Persistence of Golgi matrix distribution exhibits the same dependence on Sar1p activity as a Golgi glycosyltransferase. Traffic 2003;4:631–641. [PubMed: 12911817]

[87]. White J, Johannes L, Mallard F, Girod A, Grill S, Reinsch S, Keller P, Tzschaschel B, Echard A, Goud B, Stelzer EHK. Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. J. Cell Biol 1999;147:743–759. [PubMed: 10562278]

[88]. Young J, Stauber T, del Nery E, Vernos I, Pepperkok R, Nilsson T. Regulation of microtubule-dependent recycling at the trans-Golgi network by Rab6A and Rab6A'. Mol. Cell Biol 2005;16:162–177.

[89]. del Nery E, Miserey-Lenkei S, Falguieres T, Nizak C, Johannes L, Perez F, Goud B. Rab6A and Rab6A' GTPases play non-overlapping roles in membrane trafficking. Traffic 2006;7:394–407. [PubMed: 16536738]

[90]. Jiang S, Storrie B. Cisternal Rab proteins regulate Golgi apparatus redistribution in response to hypotonic stress. Mol. Cell Biol 2005;16:2586–2596.

[91]. Valsdottir R, Hashimoto H, Ashman K, Koda T, Storrie B, Nilsson T. Identification of rabaptin-5, rabex-5, and GM130 as putative effectors of Rab33b, a regulator of retrograde traffic between Golgi apparatus and ER. FEBS Lett 2001;508:201–209. [PubMed: 11718716]

[92]. Schmitz A, Herrgen H, Winkeler A, Herzog V. Cholera toxin is exported from microsomes by the Sec61p complex. J. Cell Biol 2000;148:1203–1212. [PubMed: 10725333]

[93]. Slominska-Wojewodzka M, Gregers TF, Walchli S, Sandvig K. EDEM is involved in retrotranslocation of Ricin from the endoplasmic reticulum to the cytosol. Mol. Biol. Cell 2006;17:1664–1675. [PubMed: 16452630]

[94]. Ackerman AL, Giodini A, Cresswell P. A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. Immunity 2006;25:607–617. [PubMed: 17027300]

[95]. Yu M, Haslam DB. Shiga toxin is transported from the endoplasmic reticulum following interaction with the luminal chaperone HEDJ/ERdj3. Infect. Immun 2005;73:2524–2532. [PubMed: 15784599]

[96]. Ghosh RN, Mallet WG, Soe TT, McGraw TE, Maxfield FR. An endocytosed TGN38 chimeric protein is delivered to the TGN after trafficking through the endocytic recycling compartment in CHO cells. J. Cell Biol 1998;142:923–936. [PubMed: 9722606]

[97]. Mallet WG, Maxfield FR. Chimeric forms of furin and TGN38 are transported from the plasma membrane to the trans-Golgi network via distict endosomal pathways. J. Cell Biol 1999;146:345–359. [PubMed: 10465644]

[98]. Bachert C, Lee TH, Linstedt AD. Lumenal endosomal and Golgi-retrieval determinants involved in pH-sensitive targeting of and early Golgi protein. Mol. Biol. Cell 2001;12:3152–3160. [PubMed: 11598199]

[99]. Puri S, Bachert C, Fimmel CJ, Linstedt AD. Cycling of early Golgi proteins via the cell surface and endosomes upon luminal pH disruption. Traffic 2002;3:641–653. [PubMed: 12191016]

[100]. Natarajan R, Linstedt AD. A cycling cis-Golgi protein mediates endosome-to-Golgi traffic. Mol. Biol. Cell 2004;15:4798–4806. [PubMed: 15331763]

[101]. Starr T, Forsten-Williams K, Storrie B. Both post-Golgi and intra-Golgi cycling affect the distribution of the Golgi phosphoprotein GPP130. Traffic. in press

[102]. Daugherty AL, Mrsny RJ. Formulation and delivery issues for monoclonal antibody therapeutics. Ad. Drug Deliv. Rev 2006;58:686–706.

[103]. Birch JR, Racher AJ. Antibody production. Ad. Drug Deliv. Rev 2006;58:671–685.

[104]. Wadia JS, Dowdy SF. Transmembrane delivery of protein peptide drugs by TAT-mediated transduction in the treatment of cancer. Adv. Drug Deliv. Rev 2005;57:579–596. [PubMed: 15722165]

[105]. Brooks H, Lebleu B, Vives E. Tat peptide-mediated cellular delivery: back to basics. Adv. Drug Deliv. Rev 2005;57:559–577. [PubMed: 15722164]

[106]. Chauhan A, Tikoo A, Kapur AK, Singh M. The taming of the cell penetrating domain of the HIV Tat: myths and realities. J. Control Release 2007;117:148–162. [PubMed: 17196289]

[107]. Gosselin MA, Lee RJ. Folate receptor-targeted liposomes as vectors for therapeutic agents. Biotech. Annu. Rev 2002;8:103–131.

[108]. Anderson R, Kamen BA, Rothberg KG, Lacey SW. Potocytosis: sequestration and transport of small molecules by caveolae. Science 1992;255:410–411. [PubMed: 1310359]

[109]. Mayor S, Rothberg KG, Maxfield FR. Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. Science 1994;264:1948–1951. [PubMed: 7516582]

[110]. Wu M, Fan J, Gunning W, Ratnam M. Clustering of GPI-anchored folate receptor independent of both cross-linking and association with caveolin. J. Memb. Biol 1997;159:137–147.

[111]. Lee RJ, Low PS. Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin *in vitro*. Biochim. Biophys. Acta 1995;1233:134–144. [PubMed: 7865538]

[112]. Lackey CA, Press OW, Hoffman AS, Stayton PS. A biomimetic pH-responsive polymer directs endosomal release and intracellular delivery of and endocytosed antibody complex. Bioconjug. Chem 2002;13:936–1001.

[113]. Yessine MA, Leroux JC. Membrane-destabilizing polyanions: interaction with lipid bilayers and endosomal escape of biomacromolecules. Adv. Drug Deliv. Rev 2004;56:999–1021. [PubMed: 15066757]

[114]. Sandvig K, van Deurs B. Membrane traffic exploited by protein toxins. Annu. Rev. Cell Dev. Biol 2002;18:1–24. [PubMed: 12142266]

[115]. Sandvig K, van Deurs B. Delivery into cells: lessons learned from plant and bacterial toxins. Gene Ther 2005;12:865–872. [PubMed: 15815697]

[116]. Johannes L, Decaudin D. Protein toxins: intracellular trafficking for targeted therapy. Gene Ther 2005;12:1360–1368. [PubMed: 15902276]

[117]. Kreitman RJ. Immunotoxins for targeted cancer therapy. AAPS J 2006;8:E532–E551. [PubMed: 17025272]

[118]. Wiseman GA, Leigh B, Erwin WD, Dominick L, Kornmehl E, Spies SM, Silverman DHS, Witzig TE, Sparks RB, White CA. Radiation dosimetry results for Zevalin radioimmunotherapy of rituximab-refractory non-Hodgkin lymphoma. Cancer 2002;94:1349–1357. [PubMed: 11877765]

[119]. Wangler C, Buchmann I, Eisenhut M, Haberkorn U, Mier W. Radiolabeled peptides and proteins in cancer therapy. Protein Pept. Lett 2007;14:273–279. [PubMed: 17346233]

[120]. Hamann PR, Hinman LM, Hollander I, Beyer CF, Lindh D, Holcomb R, Hallett W, Tsou HR, Upeslacis J, Shochat D. Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Bioconjug. Chem 2002;13:47–58. [PubMed: 11792178]

[121]. Hamann PR, Hinman LM, Beyer CF, Lindh D, Upeslaci J, Flowers DA, Bernstein I. An anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Choice of linker. Bioconjug. Chem 2002;13:40–46. [PubMed: 11792177]

[122]. Sievers EL, Linenberger M. Mylotarg: antibody-targeted chemotherapy comes of age. Curr. Opin. Oncol 2001;13:522–527. [PubMed: 11673694]

[123]. Saito G, Swanson JA, Lee KD. Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities. Adv. Drug Deliv. Rev 2003;55:199–215. [PubMed: 12564977]

[124]. Mueller BM, Wrasidlo WA, Reisfeld RA. Antibody conjugates with morpholinodoxorubicin and acid-cleavable linkers. Bioconjug. Chem 1990;1:325–330. [PubMed: 1711377]

[125]. Duncan R. Designing polymer conjugates as lysosomotropic nanomedicines. Biochem. Soc. Trans 2007;35:56–60. [PubMed: 17233601]

[126]. Seymour LW, Ferry DR, Anderson D, Hesslewood S, Julyan PJ, Poyner R, Doran J, Young AM, Burtles S, Kerr DJ. Hepatic drug targeting: phase I evaluation of polymer-bound doxorubicin. J. Clin. Oncol 2002;20:1668–1676. [PubMed: 11896118]

[127]. O'Brien AD, Tesh VL, Donohue-Rolfe A, Jackson MP, Olsnes S, Sandvig K, Lindberg AA, Keusch GT. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. Curr. Top. Microbiol. Immunol 1992;182:65–94. [PubMed: 1490392]

[128]. Sandvig, K. The Shiga toxins: properties and action on cells. In: Alouf, JE.; Popoff, MR., editors. The Comprehensive Sourcebook of Bacterial Protein Toxins. third edition. Academic Press; Paris: 2006. p. 310-322.

[129]. Acheson DWK, Calderwood SB, Boyko SA, Lincicome LL, Kane AV, Donohue-Rolfe A, Keusch GT. Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or iron-regulated promoter system. Infect. Immun 1993;61:1098–1104. [PubMed: 8432592]

[130]. Calderwood SB, Auclair F, Dohanue-Rolfe A, Keusch GT, Mekalanos JJ. Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 1987;84:4364–4368. [PubMed: 3299365]

[131]. Donohue-Rolfe A, Acheson A, Kane AV, Keusch GT. Purification of Shiga and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and the production of cross-reactive monoclonal antibodies. Infect. Immun 1989;57:3888–3893. [PubMed: 2807552]

[132]. Peter MG, Lingwood CA. Apparent cooperativity in multivalent verotoxin-globotriaosyl ceramide binding: kinetic and saturation binding studies with [^{125}I]verotoxin. Biochim. Biophys. Acta 2000;1501:116–124. [PubMed: 10838185]

[133]. Ling H, Boodhoo A, Hazes B, Cummings MD, Armstrong GD, Brunton JL, Read RJ. Structure of the Shiga-like toxin I B-pentamer complexed with an analogue of its receptor Gb_3. Biochemistry 1998;37:1777–1788. [PubMed: 9485303]

[134]. Haicheur N, Bismuth E, Bosset S, Adotevi O, Warnier G, Lacabanne V, Regnault A, Desaymard C, Amiogorena S, Ricciardi-Castagnoli P, Goud B, Fridman WH, Johannes L, Tartour E. The B subunit of Shiga toxin fused to a tumor antigen elicits CTL and targets dendritic cells to allow MHC class I-restricted presentation of peptides derived from exogenous antigens. J. Immunol 2000;165:3301–3308. [PubMed: 10975847]

[135]. LaCasse EC, Bray MR, Patterson B, Lim WM, Perampalam S, Radvanyi LG, Keating A, Stewart AK, Buckstein R, Sandhu JS, Miller N, Banerjee D, Singh D, Belch AR, Pilarski LM, Gariepy J. Shiga-like toxin-1 receptor on human breast cancer, lymphoma, and myeloma, and absence from $CD34^+$ hematopoietic stem cells: implications of *ex vivo* tumor purging and autologous stem cell transplantation. Blood 1999;94:2901–2910. [PubMed: 10515895]

[136]. Trombetta ES, Mellman I. Cell biology of antigen processing *in vitro* and *in vivo*. Annu. Rev. Immunol 2005;23:975–1028. [PubMed: 15771591]

[137]. Smith DC, Lord JM, Roberts LM, Tartour E, Johannes L. 1st class ticket to class I: protein toxins as pathfinders fro antigen presentation. Traffic 2002;3:697–704. [PubMed: 12230467]

[138]. Lee RS, Tartour E, van der Bruggen P, Vantomme V, Joyeux I, Goud B, Fridman WH, Johannes L. Major histocompatibility complex class I presentation of exogenous soluble tumor antigen fused to the B-fragment of Shiga toxin. Eur. J. Immunol 1998;28:2726–2737. [PubMed: 9754560]

[139]. Haicheur N, Benchetrit F, amessou M, Leclerc C, Falguieres T, Fayolle C, Bismuth E, Fridman WH, Johannes L, Tartour E. The B subunit of Shiga toxin coupled to full-size antigenic protein elicits humoral and cell-mediated immune responses associated with a T_h1-dominant polarization. Int. Immunol 2003;15:1161–1171. [PubMed: 13679386]

[140]. Vingert B, Adotevi O, Patin D, Jung S, Shrikant P, Freyburger L, Eppolito C, Sapoznikov A, Amessou M, Quintin-Colonna F, Fridman WH, Johannes L, Tartour E. The Shiga toxin B-subunit targets antigen *in vivo* to dendritic cells and elicits anti-tumor immunity. Eur. J. Immunol 2006;36:1124–1135. [PubMed: 16568496]

[141]. Hakomori SI. Glycosylation defining cancer malignancy: new wine in an old bottle. Proc. Natl. Acad. Sci. USA 2002;99:10231–10233. [PubMed: 12149519]

[142]. Ono M, Hakomori SI. Glycosylation defining cancer cell motility and invasiveness. Glycoconj. J 2004;20:71–78. [PubMed: 14993838]

[143]. Lingwood CA, Khine AA, Arab S. Globotriaosyl ceramide (Gb_3) expression in human tumor cells: intracellular trafficking defines a new retrograde transport pathway from the cell surface to the nucleus, which correlates with sensitivity to Verotoxin. Acta Biochim. Pol 1998;45:351–359. [PubMed: 9821866]

[144]. Farkas-Himsley H, Hill R, Rosen B, Arab S, Lingwood CA. The bacterial colicin active against tumor cells *in vitro* and *in vivo* is Verotoxin 1. Proc. Natl. Sci. USA 1995;92:6996–7000.

[145]. Arab S, Russel E, Chapman WB, Rosen B, Lingwood CA. Expression of the Verotoxin receptor glycolipid globotriaosylceramide, in ovarian hyperplasias. Oncol. Res 1997;9:553–563. [PubMed: 9507533]

[146]. LaCasse EC, Saleh MT, Patterson B, Minden MD, Gariepy J. Shiga-like toxin purges human lymphoma from bone marrow of severe combined immunodeficient mice. Blood 1996;88:1561–1567. [PubMed: 8781410]

[147]. Arab S, Murakami M, Dirks P, Boyd B, Habbard SL, Lingwood CA, Rutka JT. Verotoxins inhibit the growth of and induce apoptosis in human astrocytoma cells. J. Neuro-Oncol 1998;40:137–150.

[148]. Salhia B, Rutka JT, Lingwood C, Nutikka A, Van Furth WR. The treatment of malignant meningioma with Verotoxin. Neoplasia 2002;4:304–311. [PubMed: 12082546]

[149]. Kovbasnjuk O, Mourtazina R, Baibakov B, Wang T, Elowsky C, Choti MA, Kane A, Donowitz M. The glycosphingolipid globotriaosylceramide in metastatic transformation of colon cancer. Proc. Natl. Acad. Sci. USA 2005;102:19087–19092. [PubMed: 16365318]

[150]. Ohyama C, Fukushi Y, Satoh M, Saitoh S, Orikasa S, Nudelman E, Straud M, Hakomori S. Changes in glycolipid expression in human testicular tumor. Int. J. Cancer 1990;45:1040–1044. [PubMed: 1693599]

[151]. Ohyama C, Orikasa S, Satoh M, Saito S, Ohtani H, Fukushi Y. Globotriaosyl ceramide glycolipid in seminoma: its clinicopathological importance in differentiation from testicular malignant lymphoma. J. Urol 1992;148:72–75. [PubMed: 1613886]

[152]. Lingwood CA. Glycolipid receptors for Verotoxin and *Helicobacter pylori*: role in pathology. Biochim. Biophys. Acta 1999;1455:375–386. [PubMed: 10571026]

[153]. Hughes AK, Ergonul Z, Stricklett PK, Kohan DE. Molecular basis for high renal cell sensitivity to the cytotoxic effects of Shigatoxin-1: upregulation of globotriaosylceramide expression. J. Am. Soc. Nephrol 2002;13:2239–2245. [PubMed: 12191968]

[154]. Ohmi K, Kiyokawa N, Takeda T, Fujimoto J. Human microvascular endothelial cells are strongly sensitive to Shiga toxins. Biochem. Biophys. Res. Comm 1998;251:137–141. [PubMed: 9790920]

[155]. Newburg DS, Ashkenazi S, Cleary TG. Human milk contains the Shiga toxin and Shiga-like toxin receptor glycolipid Gb_3. J. Infect. Dis 1992;166:832–836. [PubMed: 1527419]

[156]. Arbus GS, Grisaru S, Segal O, Dosch M, Pop M, Lala P, Nutikka A, Lingwood CA. Verotoxin targets lymphoma infiltrates of patients with post-transplant lymphoproliferative disease. Leuk. Res 2000;24:857–864. [PubMed: 10996204]

[157]. Butch AW, Nahm MH. Functional properties of human germinal center B cells. Cell Immunol 1992;140:331–344. [PubMed: 1544165]

[158]. Bitzan M, Richardson S, Huang C, Boyd B, Petric M, Kamali MA. Evidence that Verotoxins (Shiga-like toxins) from *Escherichia coli* bind to P blood group antigens of human erythrocytes *in vitro*. Infect. Immun 1994;62:3337–3347. [PubMed: 8039905]

[159]. Heath-Engel HM, Lingwood CA. Verotoxin sensitivity of ECV304 cells *in vitro* and *in vivo* in xenograft tumor model: VT1 as a tumor neovascular marker. Agiogenesis 2003;6:129–141.

[160]. Tarragó-Trani MT, Jiang S, Harich KC, Storrie B. Shiga-like toxin subunit B (SLTB)-enhanced delivery of Chlorin e6 (Ce6) improves cell killing. Photochemistry and Photobiology 2006;82:527–537. [PubMed: 16613509]

[161]. Hicks S, Machamer CE. Golgi structure in stress sensing and apoptosis. Biochim. Biophys. Acta 2005;1744:406–414. [PubMed: 15979510]

[162]. Storrie, B.; Tarragó-Trani, MT.; English, S. B/B-like fragment targeting for the purposes of photodynamic therapy and medical imaging. United States Patent. 6,631,283. 2003.

[163]. Jansen KP, Vignjevic D, Boisgard R, Falguieres T, Bousquet G, Decaudin D, Dolle F, Louvard D, Tavitian B, Robine S, Johannes L. *In vivo* tumor targeting using a novel intestinal pathogen-based delivery approach. Cancer Res 2006;66:7230–7236. [PubMed: 16849571]

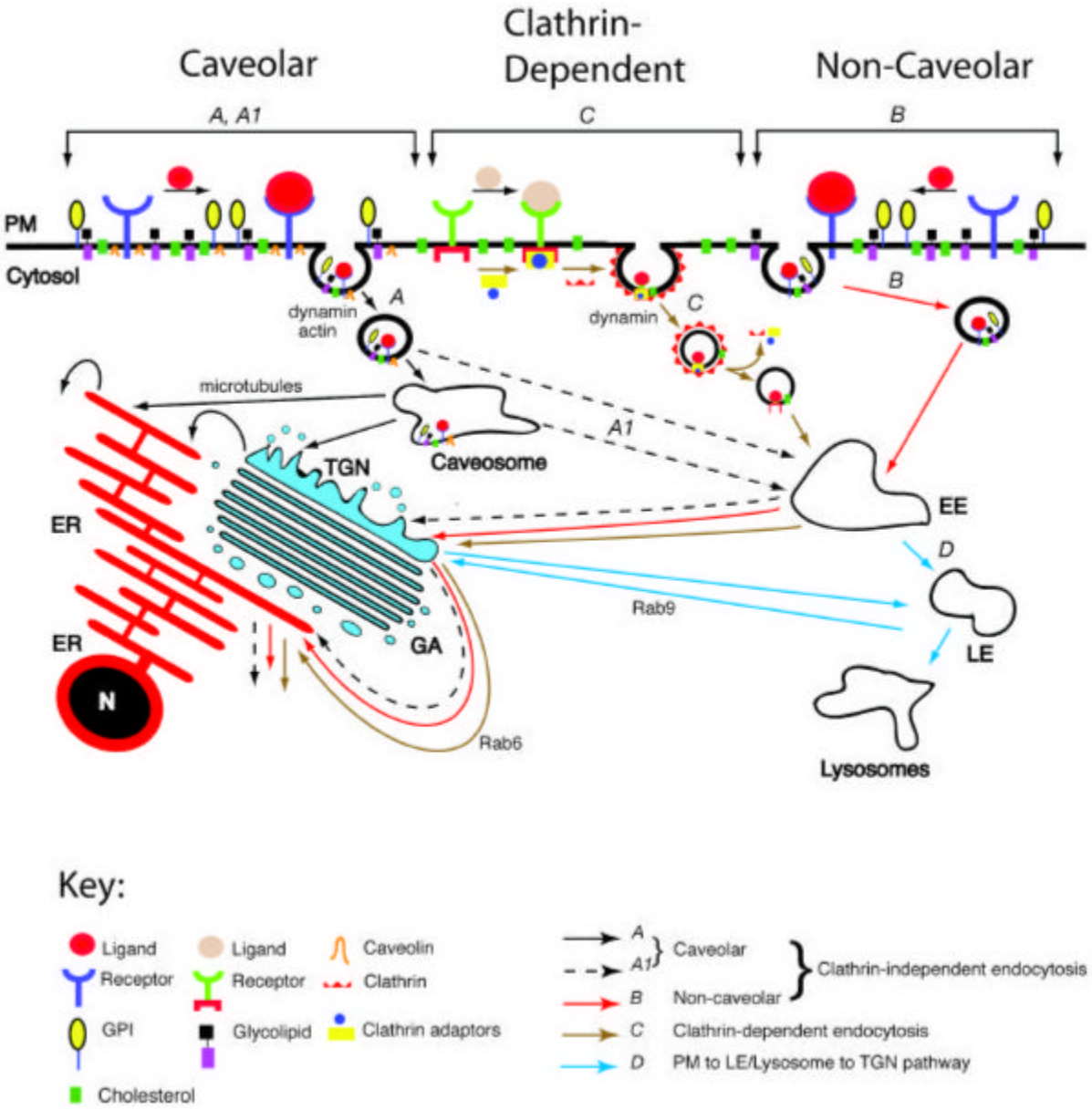

Figure 1. Different receptor-mediated endocytic mechanisms and intracellular membrane traffic pathways

A & *A1*, clathrin-independent caveolar endocytosis; *B*, clathrin-independent non-caveolar (lipid rafts) endocytosis; *C*, clathrin-dependent endocytosis; D, plasma membrane to LE/lysosomes to TGN pathway. Abbreviations: EE, early endosomes; ER, endoplasmic reticulum; GA, Golgi apparatus; GPI, glycosylphosphatidylinositol-linked proteins; LE, late endosomes; N, nucleus; TGN, trans-Golgi network.

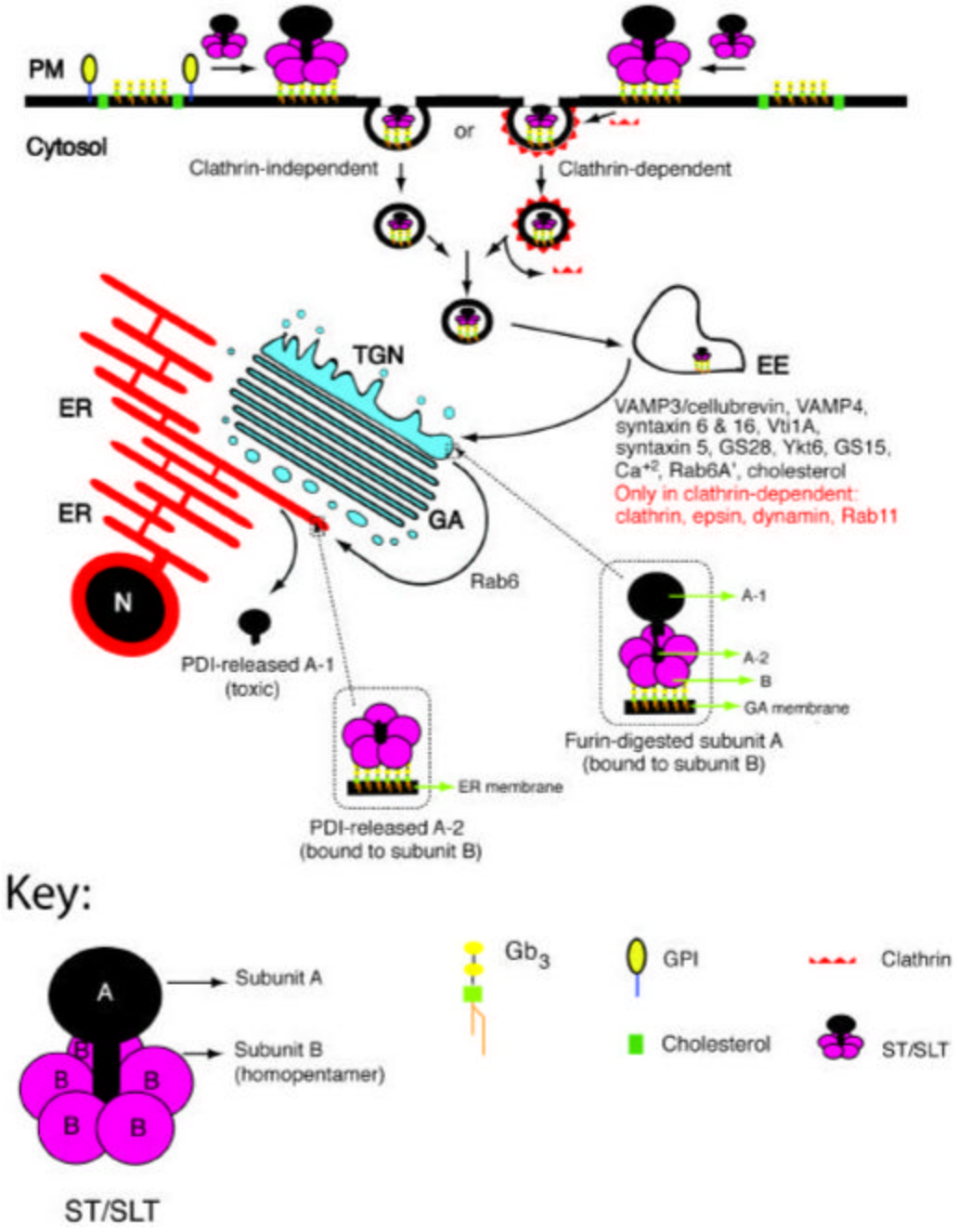

Figure 2. Modes of intracellular entrance and retrograde membrane traffic pathway of Shiga toxin and Shiga-like toxin

ST/SLT upon binding Gb_3 can enter the cell by clathrin-dependent endocytosis, and also by clathrin-independent mechanism from lipid rich, plasma membrane microdomains (lipid rafts). ST/SLT initially accumulates in EE and from there it moves in retrograde fashion to the TGN. During transport to the TGN, ST/SLT subunit A is digested by the protease furin resulting in the cleavage of subunit A into two fragments (A1 and A2) that are held together by an internal disulfide bond. ST/SLT continues its journey to the ER, where the enzyme PDI releases fragment A1 from the rest of the molecule. A1, which retains the toxic enzymatic activity, translocates then to the cytosol from the ER, allegedly using the cellular ERAD machinery.

Abbreviations: EE, early endosomes; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; GA, Golgi apparatus; GPI, glycosylphosphatidylinositol-linked proteins; N, nucleus; PDI, protein disulfide isomerase; ST, Shiga toxin; SLT, Shiga-like toxin; TGN, trans-Golgi network.

Table 1

Initial strategies to use the Shiga/Shiga-like toxins-lysosomal bypass delivery routes for therapeutic and diagnostic applications

Toxin/Subunit	Strategy	References
Shiga toxin subunit B	Delivery of antigens to MHC class 1 pathway of antigen presentation.	134,137-141
	Targeted delivery of visualizing agents (fluorescent dyes and fluor-18) to mouse spontaneous intestinal adenocarcinomas and visualization of tumors by in situ confocal laser endoscopy and positron emission tomography (PET).	163
	Targeted apoptotic killing of colon cancer cells.	149
Shiga-like toxin 1 (holotoxin)	Targeted killing of ovarian, breast, lymphoma, astrocytoma, meningioma cells.	135,144-148
	Ex-vivo purging of malignant cells from autologous stem cell grafts of breast cancer, lymphoma and myeloma patients.	135,146
Shiga-like toxin 1 subunit B	Targeted cell killing of Gb_3- expressing cells by SLTB- mediated delivery of photosensitizers.	160