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New biotechnological and nanomedicine strategies for treatment of lysosomal storage disorders

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Abstract

This review discusses the multiple bio- and nano-technological strategies developed in the last few decades for treatment of a group of fatal genetic diseases, termed lysosomal storage disorders. Some basic foundation on the biomedical causes and social and clinical relevance of these diseases is provided. Several treatment modalities, from those currently available to novel therapeutic approaches under development, are also discussed; these include gene and cell therapies, substrate reduction therapy, chemical chaperones, enzyme replacement therapy, multifunctional chimeras, targeting strategies, and drug carrier approaches.

Keywords

Lysosomal storage disorders; Enzyme replacement therapy; Chimeric enzymes; Delivery nanocarriers; Targeting and intracellular transport

Lysosomes are membrane-enclosed compartments within the cytosol of most cell types, involved in the degradation of certain lipids, sugars, proteins and nucleic acids, as well as foreign substances, such as objects and pathogens which enter cells from the extracellular milieu [1]. This function is enabled by acidic hydrolytic enzymes working in concert with small activator molecules within the lysosomal lumen. Also important for lysosomal function are transport proteins and ion pumps in the lysosomal membrane, which maintain a harsh acidic environment in this organelle [1]. Inherited deficiency of any of these lysosomal components leads to a group of diseases termed lysosomal storage disorders (LSDs) [2,3].

LSDs are therefore characterized by the accumulation of complex macromolecules within lysosomes which, due to their essential function in almost all cell types, results in pathological effects in multiple tissues and organs [2,3]. Although LSD symptoms may widely vary depending on the particular mutations inherited and the specific metabolic pathways affected, LSD clinical manifestations typically include deteriorating neurological function (50% of LSDs are associated with central nervous system (CNS) disorders), as well as pulmonary, hepatic, splenic, cardiovascular, and renal dysfunctions [2,3]. Other tissues and functions which are also often affected in LSDs are the immune system, bone, connective tissue, skeletal and cardiac muscle, dermis, and ocular function, among others [2,3].

In some cases the clinical phenotype may manifest soon after birth, causing patient mortality within the newborn period. This progression is more common for diseases strongly affecting the CNS [2,3]. In other cases the clinical manifestation may develop during infancy and progress to adulthood. These other cases are most typically characterized by non-neurological abnormalities affecting peripheral organs [2,3]. In both cases the medical conditions associated to LSDs cause high morbidity and premature mortality [2,3].

As a group, LSDs are rather prevalent genetic defects in humans and have been found to naturally occur also in several animal species [2,3]. This group comprises more than forty different life-threatening diseases [2]. It is thought to affect one in 5,000 newborns in the general population, excluding possible occurrence of patient death without prior diagnosis [4]. In particular cases, the frequency of carriers of LSD mutations in the population are as high as 1:15 or 1:80, such as in the case of Gaucher disease and type A Niemann-Pick disease in Ashkenazy Jewish descendants, which represents a rather important medical burden for these populations [2,5].

However, despite the medical and social need, the development of effective therapies for LSDs has been hampered in the past due to the low individual incidence of these diseases, which were largely considered unprofitable by the pharmaceutical industry. Although remarkable advances toward improving LSD therapeutic strategies have been made during the last decades, current treatments are generally still suboptimal and extremely costly to the health system (\$50,000–165,000/patient/year [6]).

This underscores the need to develop more effective treatments to prolong and improve the quality of life of LSD patients and their families, and to alleviate the associated economic burden. Several novel biotechnological and nanomedicine strategies to improve LSD treatment are currently under investigation.

Strategies for Treatment of Lysosomal Storage Disorders

There are limited treatments clinically available for LSDs. Symptomatic palliative therapies are readily available, but only very marginally improve the outcome of these diseases. Most classical therapeutic strategies consist of organ transplantation, such as liver transplantation (an organ largely affected in most LSDs), as well as bone marrow transplantation. While these transplantation strategies can ameliorate the disease phenotype in the reticuloendothelial system to some extent, these treatments fail to alleviate other symptoms [7,8]. A related approach is the implantation or transplantation of healthy cells (such as bone marrow or mesenchymal stem cells) obtained from normal subjects. These healthy cells, which express wild-type or normal copies of a given enzyme, are grown in culture and then implanted in patients who present deficient copies of the same enzyme, hence correcting the defect [9–11]. Although these transplantation strategies have a positive effect on the LSD clinical phenotype, this option is not available in numerous cases due to lack of compatible donors and graft-versus-host disease.

Gene therapy using viral vectors has also been under development for the last two decades as a treatment option for LSDs. Some strategies include somatic gene transfer in animal models using adenovirus, adeno-associated virus, herpesvirus and, more recently, retrovirus

and lentivirus vectors capable of integrating the exogenous gene sequences into the host genome for prolonged enzyme expression [12–17]. In many cases, similar to strategies of transplantation, gene therapies generally provide relevant correction of the enzyme deficiency in peripheral organs, mostly those of the reticuloendothelial system such as the liver, but are rather inefficient in other tissues, such as the CNS [12–17].

To address this obstacle, a considerable effort is being devoted to combinatory strategies consisting of transforming cells *ex vivo* by viral vectors to express wild-type or normal enzymes and implanting the transformed cells in the adult brain and in embryos under development (in animal models) [13,18–20]. Intracerebral administration of viral vectors of gene therapies is also under investigation. Despite representing a somewhat invasive procedure, very relevant therapeutic results associated with this practice have been obtained [21,22]. While gene therapy modalities are highly promising, their current applicability is still limited due to abnormal transport and processing of the transgenic enzymes observed in some cases and yet-unsolved safety concerns regarding gene therapies.

Another approach for treatment of LSDs is the so-called substrate reduction therapy, a strategy that employs inhibitory molecules to restrain biosynthesis of metabolites upstream of the deficient catabolic pathway that is affected in particular [23,24]. Some of these drugs are already clinically available, such as those causing partial inhibition of the glycosphingolipid biosynthetic pathway used for treatment of Gaucher disease [23,24]. Although lack of complete inhibitor specificity and long-term disruption of biosynthetic routes may cause side effects and parallel metabolic imbalances, this therapeutic strategy holds great potential. The efficiency of substrate reduction therapy in the case of neuropathic disorders is still under investigation, so the potential use of this approach for treatment of a wide range of LSDs is unclear.

Interestingly, some of these inhibitors may act also as chemical chaperones of the affected lysosomal enzymes during their biosynthesis [25,26]. Lysosomal enzymes are synthesized in the endoplasmic reticulum (ER), from which they are transported into vesicles that travel first to the Golgi apparatus and then to lysosomes [2]. Incorrect folding often results in inefficient transport of the mutated enzyme to lysosomes, with retention within upstream compartments [25,26]. The use of drug chaperones offers advantages particularly to patients with mutations that do not directly affect the enzyme activity or catalytic site but are only caused by such enzyme misfolding defects.

A related pioneering strategy has also recently emerged based on increasing the levels of cytosolic molecules that control intracellular trafficking of vesicles, such as endosomes and lysosomes. This approach facilitates the reduction of metabolites whose storage is caused by defective lysosomal transporters. In this manner, Rab9 overexpression could compensate for defects on lysosomal NPC1 or NPC2 transport proteins in type C Niemann-Pick disease, which is characterized by lysosomal accumulation of cholesterol and sphingolipids [27]. However, attaining therapeutic effects through enhancement of the intracellular levels of cytosolic molecules involved in vesicular trafficking requires the use of gene therapy or protein transduction, and therefore clinical development of this strategy is hindered by the technical obstacles of these strategies.

In the case of LSDs characterized by cholesterol accumulation, such as type C Niemann-Pick disease mentioned above, pharmacological reduction of the storage by cyclodextrin derivatives has also been shown to help attenuate the disease phenotype [28–30]. Cyclodextrins are small sugar molecules with a ring-like structure capable of extracting cholesterol from the plasma membrane and intracellular compartments [28–30]. Although the effects of these compounds on the CNS disease are still unclear, this strategy is certainly valuable and, indeed, a pilot study in two patients is being conducted at the present time.

Currently the so-called enzyme replacement therapy (ERT) is one of the most viable treatments for LDSs, and has already been used in the clinics for over a decade [31–33]. This strategy is discussed in more detail below.

Lysosomal Enzyme Replacement Therapy

The discovery of the natural transport mechanism associated with the biosynthesis of lysosomal enzymes led to a new treatment modality for these diseases: the ERT strategy (Fig. 1). As mentioned above, newly-synthesized lysosomal enzymes are initially transported in vesicular compartments from the ER to the Golgi apparatus. In this transit, the enzymes are modified by post-translational linkage of oligosaccharides containing mannose residues [34–36]. In some cases, these glycosylated enzymes are additionally phosphorylated at these mannose residues, rendering enzymes modified with mannose-6-phosphate (M6P) [37]. Then, a fraction of the enzyme population contained within these vesicles traffics intracellularly to lysosomes, while another fraction is transported to the plasma membrane and is secreted to the extracellular milieu. These extracellular enzymes can then bind either to mannose receptors (MR) present on the plasma membrane of mononuclear cells, such as monocytes and macrophages, or to the M6P receptor (M6PR) present on the plasma membrane of many other cell types [37–39]. These binding events results in recruitment of certain endocytic mediators to the plasma membrane and formation of clathrin-coated pits, where the enzyme-receptor complex is internalized and transported to endosomes and finally lysosomes [37,38].

The discovery of this phenomenon led to the idea of “cross-correction”, where the lysosomal enzyme defect in cells from LSD patients can be reverted by co-culturing said diseased cells in the presence of cells from healthy individuals [40–42]. This is due to the fact that the functional enzymes “secreted” by the healthy cells can bind to receptors on the surface of the diseased cells, being internalized and transported to lysosomes, hence correcting the enzymatic deficiency (Fig. 1). Similarly, ERT by an exogenous supply of active lysosomal enzymes to LSD patients, e.g., by infusion into the systemic circulation, provides an opportunity to overcome the enzymatic defect and attenuate the clinical phenotype in these diseases [31,32,43]. Importantly, very low amounts of the “correcting” enzyme are needed within lysosomes in order to observe a therapeutic effect: 10–20% of the endogenous levels. This is in agreement with the fact that, in the case of recessive LSDs, heterozygous individuals with one mutated allele and only a wild-type one still present a normal healthy phenotype [2].

Since the '70s, where the first attempts regarding ERT were conducted using purified natural enzymes, the biochemical and biotechnological progress has provided numerous means to keep advancing this germinal idea. Most improvements have focused on enhancing delivery of these enzymes by optimizing their glycosylation or by generating improved recombinant counterparts, several of which are currently approved for clinical use. For instance, it was soon observed that certain enzymes required post-production modifications to help in exposing mannose residues masked in their tertiary structure [44,45]. These modifications facilitated binding to cell surface receptors [44,45]. In addition, pharmacological agents that increase the expression of MRs in cells, such as dexamethasone, have been employed to improve ERTs [46]. Yet, in the case of mannose-modified enzymes, other cells are precluded from being targeted by these strategies, due to restricted expression of MRs to the reticuloendothelial system [37,47]. Alternatively, due to its broader distribution, the M6PR has been utilized for enzyme delivery to other cell types [48,49]. In some cases, however, lack of M6P residues in some recombinant enzymes leads to their rapid clearance from the circulation and suboptimal accumulation in the target organs.

The lack of effective delivery of recombinant lysosomal enzymes to all the organs affected in LSDs underlies the need to improve current strategies of ERT. As a consequence, several elegant approaches are currently focusing on methods of delivery of recombinant lysosomal enzymes by glycosylation-independent mechanisms, which hold considerable promise not only to overcome current obstacles of ERTs but also related gene therapies (including *ex vivo* gene/cell therapy) for cross-correction effects.

Chimeric Enzymes for Glycosylation-Independent Therapies

One strategy to achieve glycosylation-independent delivery of recombinant lysosomal enzymes is by generation of chimeras. These are fusion enzymes containing both a catalytically active enzymatic moiety fused to an affinity peptide. The affinity peptide serves as a targeting moiety that can be recognized by cell surface receptors in a glycosylation-independent manner (Fig. 2). This is the case for fusion enzymes targeted to the M6PR, those targeted to endocytic receptors distinct from the ones utilized by endogenous lysosomal enzymes, and those taking advantage of protein transduction domains.

For instance, targeting of the M6PR that is involved in endocytosis of lysosomal enzymes can be achieved in a glycosylation-independent manner by capitalizing on the insulin-like growth factor II (IGF-II), which binds to a different domain of the same receptor [50]. Based on this principle, a chimeric enzyme was created, containing recombinant β -glucuronidase (the enzyme deficient in cases of mucopolysaccharidosis -MPS- VII) fused to a peptide derived from IGF-II [51] (Table 1 and Fig. 2). Such a chimera efficiently targeted M6PR, followed by clathrin-mediated endocytosis and lysosomal delivery, as in the case of M6P-containing enzymes [51].

In skin fibroblasts from MPS VII patients and the MPS VII mouse model, binding of the aforementioned chimeric enzyme to the M6PR was enhanced as compared to the enzyme lacking the targeting peptide. Interestingly, the M6P residues present on the enzyme and the

IGF-II counterpart contributed to targeting the corresponding domains on M6PR, as both excess free IGF-II or M6P partially inhibited binding [51]. Yet removal of M6P residues in this fusion protein still rendered efficient binding and endocytosis, indicating that glycosylation-independent targeting of recombinant enzymes and/or enhanced affinity of glycosylated enzymes to their natural receptors are promising strategies to improve lysosomal ERT.

Similarly, these chimeric enzymes can be designed to target alternative endocytic receptors present on the cell surface, such as the case of α -L-iduronidase or α -glucosidase, the lysosomal enzymes deficient in cases of Hurler/Scheie syndrome or MPS I and Pompe disease, respectively. These enzymes have been synthesized as recombinant proteins fused to a peptide derived from the receptor associated protein, RAP [52] (Table 1 and Fig. 2). RAP is located in the endoplasmic reticulum, where it binds as a chaperone to several LDLR family receptors during their biosynthesis, preventing premature association to their physiological ligands [53]. Also, cell surface LDLR family receptors targeted by RAP are internalized by cells via clathrin-dependent endocytosis, similar to receptors of the endogenous lysosomal enzymes [54].

RAP-derived chimeras of α -L-iduronidase or α -glucosidase showed a significant enhancement of uptake into skin fibroblasts isolated from patients, rat glioma cell cultures, and mouse myoblasts [52]. Uptake was prevented by free RAP and mediated by LDLR-related protein 1 or LRP1, apoER2, VLDLR, and megalin, all proteins of the LDLR family [52]. Thus, RAP provides both glycosylation-independent targeting and the possibility of delivering recombinant enzymes to a variety of cell surface receptors.

In addition to targeting to endocytic receptors, fusion enzymes containing protein transduction domains, such as peptides derived from the HIV transcription factor Tat, have been designed to improve delivery of recombinant lysosomal enzymes. In contrast to the specific receptor-targeting approaches described in the previous paragraph, this strategy is based on charge-mediated electrostatic interactions of Tat with membrane phospholipids and polysaccharides [55,56]. Such interactions then facilitate internalization of the enzyme within cells [55,56]. Although the mechanism of Tat internalization is still controversial, it is believed to occur as a combination of various routes, including passive uptake within vesicles that mediate endocytosis of extracellular ligands and plasma membrane components and/or direct penetration through the plasmalemma [55,56].

Several works have focused on gene and enzyme therapies using recombinant lysosomal enzymes fused to Tat peptides, including β -glucuronidase, glucocerebrosidase, and galactocerebrosidase for treatment of MPS VII, Gaucher, and Krabbe diseases, respectively (Table 1 and Fig. 2). Tat fusion enzymes have been designed for efficient expression and secretion in eukaryotic *Sf9* insect cells [57]. These enzymes have also been shown to enter a variety of cell types in culture (including alveolar epithelial cells and fibroblasts) and to traffic to lysosomal compartments via both M6PR-dependent and independent pathways [58–60]. Interestingly, β -glucuronidase-Tat expressed from adenoviral vectors administered to mice was found in non-transduced cells [59]. This finding indicates that the chimera was secreted from transduced cells, resulting in a “cross-correction” effect [59]. The efficiency

of ERT strategies using Tat fusion enzymes has not been established yet in animal models. Whether the lack of specificity of Tat-derived peptides for any specific cell type may improve broad biodistribution of the administered enzyme or simply result in enhanced uptake by the clearance organs is still unknown. However, Tat-targeting strategy holds considerable promise for the improvement of lysosomal ERT.

These chimeric lysosomal enzymes fused to targeting peptides display enhanced receptor-mediated and/or receptor-independent uptake by cells in culture and improved delivery to certain organs, mainly those of the reticuloendothelial system, in animal models. However, the CNS remains a challenging target for most of these strategies. The need for alternative approaches to improve ERT biodistribution to this tissue is compelling.

Brain Targeting of Lysosomal Enzymes

As mentioned above, recombinant enzymes injected into the circulation gain rapid access to cells in certain organs, such as the liver and spleen. This is due to passive passage through the large micron-size gaps between the endothelial cells in the wall of the blood vessels irrigating these organs [61–63]. This is in contrast to the CNS, a main target for LDS therapeutic intervention, where endothelial cells form a continuous layer with sealed junctions between the adjacent cells [61–63]. As a consequence, classical ERT strategies are suboptimal in attenuating the strong neurological deterioration associated to more than half of LSDs [2].

Transient disruption by hyperosmolar solutions, solvents, adjuvants, ultrasound, and surgical interventions (intracerebroventricular or intracerebral delivery) have been classically used to traverse the blood-brain barrier, but these approaches are invasive, toxic, and/or fail to provide penetration into the tissue parenchyma [64–68]. An alternative strategy consists of capitalizing on certain cell receptors expressed on brain endothelial cells. These receptors mediate transport of substances across the blood-brain barrier via endocytic vesicles that travel from the luminal to the basal cell surface for delivery into the brain (Table 1 and Fig. 3). This route is known as transcellular transport or transcytosis [69–72]. In the brain, this transport is regulated mainly by receptors activating clathrin-mediated endocytic pathways [70] that are inducible by targeting recombinant lysosomal enzymes to these receptors. This is the case for strategies that target the M6P, transferrin, or insulin receptors (Table 1 and Fig. 3).

For instance, β -glucuronidase (the enzyme deficient in cases of MPS VII) and phosphorylated sulfaminase (the enzyme deficient in cases of MPS IIIA) have been shown to be transported to the brain of animal models for these diseases, which is mediated by the M6PR [73]. However, brain influx of the enzymes injected intravenously was only efficient in 2-day-old neonates [73]. Brain transport in 8-week-old adult mice was negligible due to the fact that transcytosis mediated by brain M6PR is only active early after birth and quickly downregulated developmentally [73].

In contrast to M6PR, the insulin receptor and transferrin receptor are active in adult brain endothelia cells, providing transport across the blood-brain barrier (Figs. 2 and 3). It was recently shown that α -L-iduronidase fused to a peptide derived from an antibody against the

human insulin receptor accumulated in the brain of adult *Rhesus* monkey following intravenous administration [74]. This chimeric enzyme was also capable of attenuating about 70% of the glycosaminoglycan accumulation typical in MPS I. A similar strategy recently tested in the MPS I mouse model demonstrated that delivery of a plasmid codifying for a fusion protein comprising transferrin and α -L-iduronidase was also effective in attenuating glycosaminoglycan in the mouse cerebellum [75] (Figs. 2 and 3).

Polymer and Liposomal Formulations for Delivery of Lysosomal Enzymes

Enhanced delivery of chimeric lysosomal enzymes by means of fusion targeting peptides may improve the efficacy of current treatments by ERT. The success of this approach relies on the stability and activity of the chimeric protein, which greatly varies among different lysosomal enzymes and particular constructs. Fusion constructs often fail to render active enzymatic moieties. In addition, given that LSDs are chronic maladies, recurrent intravascular injection of recombinant enzymes, and likely their chimeric counterparts, induces immunological effects, such as generation of blocking antibodies that hinder the long term efficacy of ERT.

Immunological effects that influence the enzymatic activity are observed in ERT administration by intravenous infusion of recombinant α -galactosidase in Fabry disease patients [76]. Similarly, intravenous injection of recombinant arylsulfatase A in the animal model for metachromatic leukodystrophy results in the production of antibodies against the recombinant enzyme [77]. These antibodies hinder clearance of sulfatide, the enzyme substrate accumulated in this disease, even without inhibiting the enzyme activity [77]. This effect is due to antibody blockage of the enzyme binding to M6PR, resulting in low endocytosis and delivery to lysosomes [77]. Related effects have been reported for a number of other LSDs [78].

Thus, strategies to prolong the circulation of such recombinant enzymes by masking them from recognition by the immune system may enhance the enzyme accumulation in the target organs and protect them from rapid inactivation while attenuating potential immunological reactions. This can be achieved by coupling recombinant lysosomal enzymes to polyethylene glycol or PEG, which is known to establish interactions with water molecules generating a hydration layer, “protective” against recognition by the complement system and immune cells. For instance, in a mouse model of LSD mimicked by lysosomal accumulation of dextran, PEG-modified dextranase reverted dextran storage in the liver with higher efficiency than its non-PEG counterpart, likely due to enhanced delivery and/or prolonged stability of the enzyme [79] (Table 2).

Additionally, encapsulation of recombinant lysosomal enzymes within liposomes was explored in the past as a means to improve their biodistribution (Table 2). Liposomal formulations of β -glucuronidase showed enhanced enzyme uptake in the liver [80]. Interestingly, both distribution and retention of the enzymatic activity could be modulated, at least in part, by varying the liposomal charge. For instance, although β -glucuronidase loaded in either negatively or positively charged liposomes accumulated efficiently in the liver and, at some extent, in the kidneys of mouse models, the enzyme delivered by

positively charged liposomes displayed a significantly prolonged activity (11 days versus 2 days), whereas negatively charged liposomes trafficked more effectively to lysosomes [80].

Treatment of mice with a model enzyme (dextranase) coupled to PEG and encapsulated into liposomes rendered enhanced delivery and prolonged activity in hepatic lysosomes, likely by bypassing immunorecognition [79]. In contrast, recognition of a therapeutic formulation by cells of the immune system is required for efficient treatment in certain diseases, e.g., polymorphonucleocytes for treatment of Batten disease. In this case, liposomes were adapted to this delivery modality by loading of recombinant lysosomal enzymes in liposomes coupled to aggregated immunoglobulins or apolipoproteins [81,82] (Table 2).

Entrapment of recombinant lysosomal enzymes in biocompatible polymers may provide a means of improving the therapeutic outcome in refractory areas that are difficult to access from the circulation. One example of this strategy is local implantation of such polymers, for controlled and prolonged enzyme release. This idea was explored in cell culture by entrapping recombinant glucocerebrosidase in alginate microspheres designed for sustained release of the enzyme, which may be beneficial for ERT of bone tissue in the case of Gaucher disease [83] (Table 2).

Apart from these seminal works mostly conducted in the late '70s, few other approaches to improve ERTs by using carriers to control the enzyme biodistribution have been attempted, despite the potential utility of this strategy.

ICAM-1-Targeted Nanocarriers for Delivery of Lysosomal Enzymes

In the last decades a great effort has been devoted to develop biomaterials and nanotechnologies to improve delivery of therapeutic agents. For example, researchers have developed sub-micrometer size carriers (nanocarriers) that can be designed to increase the bioavailability of therapeutic compounds, protect them from degradation, control their circulation time and release rate, and provide site-specific targeting to the sites in the body affected by disease, altogether improving the therapeutic outcome while minimizing secondary effects [84–91]. Many of these macromolecular nano-assemblies (apart from phospholipid liposomes) have been designed for this purpose, including carbon nanostructures, branched dendrimers, and amphiphilic polymers formulated as self-assembled micelles or polymer particles [84–91].

The nanocarrier size, which ranges from ~100–300 nm, is adequate to avoid passive diffusion through tissues and renal clearance, while being compatible with physiological transport mechanisms. The nanocarrier surface can be modified with affinity moieties (antibodies, peptides, sugars, aptamers) to provide recognition of markers expressed by cells at the disease sites. Selection of markers involved in biological transport pathways further provides delivery to compartments within the cell interior and/or across cell barriers. For instance, nanocarriers can be targeted to cell-surface receptors involved in endocytic pathways leading to lysosomal transport or transport across the cell body (transcytosis) for penetration into the brain [70,92–95].

In theory, the concept of ERT assisted by targeted nanocarriers can be implemented by injecting said formulations in the systemic circulation, from where the recombinant lysosomal enzymes can be distributed to the target organs and cells, reaching lysosomes at the sub-cellular level. In this regard, given that LSDs are characterized by multi-organ dysfunction, an ideal target for nanocarrier-assisted lysosomal ERT would be a molecule expressed on all of these organs.

Intercellular adhesion molecule 1 (ICAM-1) (Fig. 4), an immunoglobulin-like transmembrane glycoprotein that mediates adhesion of fibrinogen and leukocytes to inflammation sites [96–98], seems to be a particularly unique target for lysosomal ERT interventions. ICAM-1 is expressed by most LSD target cells, including vascular endothelial cells of vessels irrigating all organs, cells of the reticuloendothelial system, leukocytes, epithelial cells, glial cells, neurons, muscle cells, and others [96]). Additionally, ICAM-1 is accessible via intravascular, intratracheal, or intracerebral routes [95]. ICAM-1 expression is enhanced by many pathological factors present in LSDs, such as inflammation, thrombosis, atherosclerosis, oxidative stress, and metabolic imbalance [96,99,100]. Finally, ICAM-1 targeting blocks leukocyte anchoring, providing anti-inflammatory benefits [101,102]. In some particular cases, such as that of type A and B Niemann-Pick disease due to deficient acid sphingomyelinase (ASM), ICAM-1 engagement increases enzyme activity [103], which may benefit patients with mutations rendering residual enzyme activity.

However, ICAM-1 is not considered an endocytic receptor, a feature required for intracellular (lysosomal) delivery of lysosomal recombinant enzymes for treatment of LSDs. In this regard, targeting of lysosomal enzymes to non-endocytic receptors has not been explored due to logical skepticism on the translational impact of this approach. Yet, in some cases, binding of artificial ligands to non-internalizable cell surface molecules may modify their structure and/or function and favor endocytosis, necessary in the context of delivery of lysosomal ERT. This is the case for ICAM-1 (Fig. 5): although binding of its natural ligands (leukocytes) or antibodies to ICAM-1 do not induce endocytosis, a variety of cell types efficiently internalize artificial multivalent ligands, such as those generated by conjugation of biotinylated anti-ICAM with streptavidin or by adsorbing anti-ICAM on the surface of polystyrene beads or polymer nanocarriers [104–107]. Uptake of such multivalent ligands targeted to ICAM-1 is mediated via a unique pathway: cell adhesion molecule or CAM-mediated endocytosis is distinct from clathrin- and caveolar-mediated pathways, macropinocytosis, and phagocytosis [107–109] (Fig. 5).

The molecular partners, signal cascades and cytoskeleton changes key to CAM-endocytosis have been defined for the most part [107–110] (Fig. 5). Recently, engagement of only certain extracellular domains of CAMs, preferentially those involved in interaction with their natural ligands, was found to transmit outside-in signals leading to plasmalemma invaginations and endocytosis [110,111]. Multivalent engagement of these extracellular domains of CAMs results in tyrosine phosphorylation in their cytosolic domain, which leads to phosphorylation and activation of signaling molecules, e.g., small GTPases that regulate actin polymerization [110].

In contrast to classical clathrin and caveolar pathways, which are characterized by vesicles ~100–150 nm and ~50–80 nm in diameter, respectively, CAM-endocytosis is effective both in cell cultures and in mice even in the case of objects presenting a wide variety of geometries, e.g., anti-ICAM particles from 100 nm to 5 μ m size, spherical or elongated and even irregular in shape [104,107,112]. This notorious capacity is similar to that of phagocytosis, yet phagocytosis is regulated differently and is restricted to specialized cells of the immune system, while CAM-endocytosis operates in all the cells tested to date.

ICAM-1 and CAM-endocytosis are excellent candidates for developing strategies aimed at targeting therapeutics to disease sites and transporting them into cells by using drug delivery carriers. ICAM-1 is overexpressed at disease sites during pathology, it sustains endocytosis of a variety of particulate materials, and key factors regulating ICAM-1-mediated intracellular transport are increasingly known. Our group and others have successfully used ICAM-1 targeting for delivery of isotopes and contrast probes [113,114], biosensors [115], therapeutics [104,106,108,109,116], liposomes and polymer nanocarriers [105,117–119] in cell cultures and animals. Although a detailed study is still needed, this strategy is relatively safe: in cell culture and *in vivo* ICAM-1-targeted polymer nanocarriers do not affect the endothelial permeability or cell junctions, or cause changes in gross histology, cell morphology, division, endocytosis and lysosomal transport, and overall cell viability [108,112,118].

Importantly, ICAM-1 recycles to the plasma membrane following endocytosis providing sustained binding and internalization of circulating anti-ICAM nanocarriers [108], whereas a significant fraction of the internalized nanocarriers traffic intracellularly from early endosomes to lysosomes [108,109,116] (Fig. 5). From the standpoint of other therapies, this represents a limitation since degradation of the cargo shortens the duration of the therapeutic effects [108,109,116]. Yet, in the context of lysosomal ERT, lysosomal destination is an ideal feature of ICAM-1 delivery systems, providing degradation of the targeting moiety and carrier while delivering the therapeutic cargo to its destination. Furthermore, the rate of endocytosis and lysosomal transport of CAM-targeted nanocarriers can be fine-tuned via multiple design parameters, including the molecular epitope targeted by the carriers [111], the carrier size and shape [112], and auxiliary drugs modulating the cell machinery involved in endocytic vesicular transport [109,116].

As an example of the concept of nanocarrier-assisted treatment of LSDs, we have investigated basic parameters of ICAM-1 targeting in cell cultures and mouse models regarding ERT for type A and B Niemann-Pick disease (Fig. 6). As mentioned above, this disease is due to a genetic deficiency of acid sphingomyelinase (ASM), which leads to aberrant storage of sphingomyelin within lysosomes and affects mainly the CNS (type A) or the lungs, liver and spleen (type B) [5]. We co-coated anti-ICAM and recombinant human ASM (designed and kindly provided by our collaborator Dr. Edward Schuchman) by adsorption on the surface of 100 nm model polystyrene particles or biodegradable poly(lactic-co-glycolic acid) nanocarriers, an FDA approved material [106,112,118]. These carriers are comprised of the therapeutic cargo (ASM) and an ICAM-1-targeting vector (anti-ICAM) in a multivalent complex capable of targeting different organs in the body [118] to induce CAM-mediated endocytosis and lysosomal transport [106,112]. Given that

lysosomal enzymes are only fully active in the acidic lysosomal environment, lysosomal enzymes loaded on the carrier surface can be considered “pro-drugs” until they reach the lysosomes. Therefore, their encapsulation within nanocarrier particles is not necessary. Exposure of recombinant enzymes on the carrier surface renders fast recovery of activity upon delivery to lysosomes [106]. In theory, ASM on the carrier surface may attenuate high sphingomyelin levels in the vascular wall at atherosclerotic sites in Niemann-Pick disease patients. In these areas ICAM-1 is overexpressed and the microenvironment is acidic [120].

Our recent work in animal models (Fig. 6) demonstrated that ICAM-1-targeted nanocarriers markedly enhance (up to 100 fold in some cases) ASM targeting to and accumulation in all organs, including liver, spleen, heart, kidney, lung [118] and, as observed most recently, the brain (Muro, unpublished results). ASM targeting is abolished by anti-ICAM, not M6P [106], and is relative to ICAM-1 expression level: e.g., accumulation of anti-ICAM/ASM nanocarriers in ASMKO lungs doubles that of in control mice [118], illustrating the property of this strategy to favor delivery to disease sites.

Furthermore, in cell culture (Fig. 6) anti-ICAM/ASM nanocarriers efficiently enter cells by CAM endocytosis (bypassing the clathrin pathway associated to lysosomal enzymes) and efficiently transport the enzyme to lysosomes [106,112]. Within lysosomes, anti-ICAM is degraded while ASM is processed to a lower molecular-weight form similar to the native enzyme, and remains stable for long periods of time [106]. Notably, although non-targeted ASM provides partial degradation of sphingomyelin, ASM delivered by anti-ICAM nanocarriers restores lysosomal sphingomyelin control levels [106], indicating strategic potential to improve lysosomal ERT.

We are optimizing this approach for pre-clinical testing of its potential utility and safety regarding ERT for LSDs. More biocompatible means to target ERTs to ICAM-1 via small affinity peptides derived from either anti-ICAM antibodies or isolated by phage-display are currently under way. The application of this strategy as a general platform for lysosomal ERT beyond type A and B Niemann-Pick disease is also being investigated.

Conclusion

In summary, a variety of biotechnological and nanomedicine approaches have been and are being developed to cope with the multiple medical syndromes associated with genetic LSDs. These include gene and cell therapies, substrate reduction therapy, the use of chemical chaperones and small drugs, and ERTs. Strategies of targeting of therapeutic genes and recombinant enzymes using genetic engineering and recombinant technology for generation of multifunctional chimeras presenting both enzymatic activity and targeting capability, are promising as means to improve treatment of these diseases. New advances in nanotechnologies for controlled circulation, organ targeting, effective intracellular trafficking, and release of lysosomal enzymes offer additional opportunities to translate these therapeutic modalities into clinically relevant treatments for LSDs. All these approaches offer valuable advantages, for which it is likely that additive and/or synergic combinatory therapies will soon arise as the future of LSD treatment.

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Figure 1. Lysosomal enzyme transport

After their biosynthesis in the endoplasmic reticulum (ER), lysosomal enzymes are transported to the Golgi apparatus where they are modified by addition of sugar residues and, in some cases, phosphorylation. Certain enzyme residues, such as mannose 6 phosphate (M6P) are then recognized by M6P receptors (M6PR), which mediate intracellular trafficking to lysosomes. Lysosomal enzymes are also “secreted” to the extracellular milieu, where they can bind to M6PR on the cell surface of the same cell or a different cell. This mediates clathrin-mediated uptake and endocytic transport to lysosomes. This pathway constitutes the foundation for delivery of recombinant enzymes in replacement therapy protocols.

Figure 2. Chimeric lysosomal enzymes

To improve delivery of replacement therapies for treatment of lysosomal storage disorders, the recombinant enzymes can be produced as multifunctional fusion proteins or chimeras comprising the enzymatic module and an affinity peptide, which provides affinity to the cell surface. These peptides can be designed to target receptors of endogenous lysosomal enzymes, such as the mannose 6 phosphate receptor (M6PR), which can be targeted by M6P and insulin-like growth factor II (IGF II). Chimeric enzymes can also be targeted to alternative receptors of endocytosis, such as low density lipoprotein receptor (LDLR) family, which is targeted by receptor associated protein (RAP), transferrin receptor (TfR), targeted by transferrin (Tf), or insulin receptor (InsR), targeted by a peptide derived from an antibody to InsR (anti-InsR). These fusion enzymes can also be addressed to negatively charged domains of the plasma membrane, which can be targeted by Tat peptides. Chimeric enzymes targeted by these peptides can enter cells and traffic to lysosomes via clathrin-mediated endocytosis (those targeted to the receptors described above) or unspecific endocytosis (those targeted by Tat).

Blood vessel lumen

Transcellular route

Paracellular route

Figure 3. Brain targeting of recombinant lysosomal enzymes

To improve delivery of replacement therapies to the brain in the case of lysosomal storage disorders affecting the central nervous system, recombinant enzymes can be designed to target receptors involved in transcellular transport of substances across the endothelium in the blood-brain barrier. This transport mediated by vesicular transcytosis is relatively safe, as it does not cause opening of the junctions between adjacent endothelial cells maintaining the permeability barrier (the paracellular route). M6PR = mannose 6 phosphate receptor; TfR = transferrin receptor; InsR = insulin receptor.

Figure 4. ICAM-1 structure

ICAM-1 is characterized by a long glycosylated extracellular region with five immunoglobulin-like domains (D1 to D5), a transmembrane region, and a short cytosolic tail. The extracellular domains mediate interaction with leukocyte β_2 integrins (LFA and Mac-1) and fibrin(ogen). The cytosolic domain mediates signal transduction upon engagement of the extracellular domains. ICAM-1-mediated signaling includes tyrosine phosphorylation of SHP-2 helped by Grb2, PKC activation, phosphorylation of Src kinases, signaling through MAP kinases, Rho-dependent remodeling of the cytoskeleton, activation of cortactin and focal adhesion kinase (FAK), and interaction with α -actinin, ezrin and moesin, and the microtubule-related protein β -tubulin [95].

Figure 5. ICAM-1, CAM-mediated endocytosis and lysosomal transport

CAM-endocytosis is induced upon ICAM-1 engagement by multivalent ligands, such as 100 nm polystyrene or PLGA nanocarriers coated by anti-ICAM. This pathway involves PKC, Src kinases, and Rho-dependent kinase (ROCK) and formation of actin stress fibers. ICAM-1 interacts with the amiloride-sensitive, sodium/proton exchanger NHE1, which contributes to cell surface deformability and is an adaptor of the cytoskeleton. Dynamin is involved in budding of the nascent vesicles. These events contribute to the progression of membrane invaginations and intracellular vesicular transport. After internalization, ICAM-1/anti-ICAM carrier complexes traffic to endosomes that are positive for EEA1 and PKC-regulated NHE6. ICAM-1 recycles then to the plasmalemma, and anti-ICAM nanocarriers traffic to LAMP1-positive lysosomes.

Figure 6. Enzyme replacement therapy by ICAM-1-targeted nanocarriers

Model polymer nanocarriers (FITC-labeled polystyrene, 100 nm diameter) were coated by surface absorption with recombinant acid sphingomyelinase (ASM, Schuchman – Mount Sinai School of Medicine) and monoclonal anti-ICAM or control IgG. Upon injection *in vivo* in mice, only ICAM-1-targeted carriers (lower panel, left), but not control IgG carriers (upper panel, left), showed efficient binding to the vasculature irrigating all organs (mesentery is shown) [117]. At the cellular level, incubation of skin fibroblasts from type B Niemann-Pick disease patients with non-fluorescent carriers for 3 h (lower panel, right) significantly attenuated lysosomal sphingomyelin (labeled by a green-fluorescent BODIPY derivative, upper panel, right) [105].

Table 1

Lysosomal enzyme fusion proteins

Disease	Enzyme	Tag	Cell culture	In vivo	Target	Uptake pathway	M6PR independent	M6PR dependent	Ref.
Gaucher	Glucocerebrosidase	Tat	Y	N	(-) membrane	Unspecific	N/D	N/D	[56,57]
Krabbe	Galactocerebrosidase	Tat	Y	N	(-) membrane	Unspecific	Y	Y	[59]
MPS I	α -L-Iduronidase	Anti-InsR	Y	Y	InsR	Clathrin	N/D	N/D	[73]
MPS I	α -L-Iduronidase	Tf	N	Y	TfR	Clathrin	N/D	N/D	[74]
MPS I	α -L-Iduronidase	RAP	Y	N	LDLR family	Clathrin	Y	Y	[51]
MPS VII	β -Glucuronidase	IGF-II	Y	Y	M6PR	Clathrin	Y	Y	[50]
MPS VII	β -Glucuronidase	Tat	Y	Y	(-) membrane	Unspecific	N/D	N/D	[58]

MPS = mucopolysaccharidosis; InsR = insulin receptor; Tf = transferrin; TfR = transferrin receptor; LDLR = low density lipoprotein receptor; M6PR = mannose 6 phosphate receptor; (-) = negatively charged molecules; Y = yes; N = no; N/D = not determined

Table 2

Lysosomal enzyme carriers

Disease	Enzyme	Carrier	Cell culture	In vivo	Target	Uptake pathway	M6PR independent	M6PR dependent	Ref.
LSD model	Dextranase	PEG, liposomes	N	Y	N/A	N/D	N/D	N/D	[78]
LSD model	Horseshoe peroxidase	IgM liposomes	Y	N	IgMR	Phagocytosis	N/D	N/D	[81]
Batten	ATP-synthase c	IgG & apoE liposomes	Y	N	FcR, apoER	Phagocytosis	N/D	N/D	[80]
Gaucher	Glucocerebrosidase	Alginate microspheres	Y	N	N/A	N/D	N/D	N/D	[82]
MPS VII	β -Glucuronidase	(-) & (+) liposomes	N	Y	N/A	N/D	N/D	N/D	[79]
NPD-AB	Acid sphingomyelinase	Anti-ICAM PLT & PLGA particles	Y	Y	ICAM-1	CAM endocytosis	Y	N	[105,111,117]

LSD = lysosomal storage disorder; MPS = mucopolysaccharidosis; NPD-AB = type A and B Niemann-Pick disease; PEG = polyethylene glycol; PLT = polystyrene; PLGA = poly(lactic-co-glycolic acid); IgMR = IgM receptor; FcR = Fc receptor; apoER = apoE receptor; CAM = cell adhesion molecule; (-) = negatively charged; (+) = positively charged; Y = yes; N = no; N/A = not applicable; N/D = not determined.