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Small Molecules: Substrate Inhibitors, Chaperones, Stop-Codon Read Through, and Beyond

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Abstract

Lysosomal storage disorders are rare genetic disorders due to deficient lysosomal activity, which leads to progressive accumulation of nonmetabolized substrates. Patient's clinical outcomes have significantly improved since the advent of enzyme replacement therapy, even though this therapeutic approach presents important limitations, such as immune reactions, low bioavailability of recombinant enzymes, and incapability to reach the central nervous system. New strategies based on gene therapy or small molecules have been proposed and tested as an alternative to enzyme replacement therapy or to complement it. Small molecules are orally administrated, no antigenic compound that can diffuse across cell membranes and distribute in steady-state concentrations, also reaching the central nervous system. Substrate reduction therapy, pharmacological chaperones, and stop-codon read-through enhancers are small molecules currently available for the treatment of lysosomal storage disorders. This article describes the characteristics of this class of compounds and the possible strategies to improve their efficiency in future development.

Keywords

lysosomal storage disorders, substrate reduction therapy, pharmacological chaperones, stop-codon read through, cotreatment with enzyme replacement therapy

Introduction

Lysosomal storage disorders (LSDs) are a group of more than 50 different diseases due to a functional deficiency of a lysosomal protein (ie, acidic hydrolases, activator transporters, or nonlysosomal proteins necessary for lysosomal function), which leads to the accumulation of a variety of substrates, such as glycosaminoglycans (GAGs), glycosphingolipids, glycogen, oligosaccharides, cholesterol, peptides, and glycoproteins.[1] A broad clinical presentation is associated with LSDs, which includes visceral, ocular, hematological, skeletal, and neurological manifestations. These outcomes eventually affect lifespan, as well as physical and intellectual performance of the patients, determining an important challenge in terms of public health, social rights, and economic costs for the society. Even if LSDs are considered singularly as rare diseases (incidence ranging between 1:7000 and 1:1 000 000), collectively these pathologies are as frequent as 1:5000 to 1:7000 live births.[2]

Nowadays, the most frequent LSDs (ie, Gaucher disease [GD], Fabry disease [FD], Pompe disease [PD], mucopolysaccharidosis [MPS], type I, II, IV, VI, and acid lipase deficiency) are treated by enzyme replacement therapy (ERT) or substrate reduction therapy (SRT).[3]

The ERT consists of periodic intravenous administration of the recombinant form of the human wild-type enzyme, which can be internalized by the cells with deficient lysosomal activity, through the mannose 6-phosphate receptor (MP6). The ERT has been used in the clinic for around 15 years with relevant benefits in patients' quality of life, allowing, as an example, the normalization of hematologic parameters and organ volumes in GD or the decrease in neuropathic pain crisis

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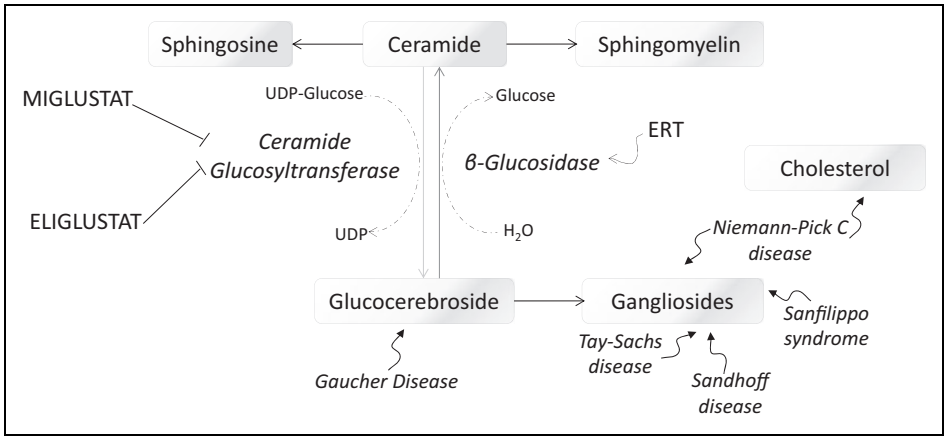


Figure 1. Mechanism of action of SRT drugs for GD and other LSDs. The SRT drugs like miglustat and eliglustat are competitive inhibitors of ceramide glucosyltransferase. By reducing glucocerebroside concentration, these drugs facilitate the degradation of the substrate by the β-glucosidase, the enzyme affected in GD. Glucocerebroside is a precursor of gangliosides accumulated in TSD, MPS III, SD, and Niemann-Pick C diseases. ERT enhances the activity of β-glucosidase. GD indicates Gaucher disease; LSD, lysosomal storage disorder; SRT, substrate reduction therapy; TSD, Tay-Sachs disorder; MPS III, mucopolysaccharidosis type III; SD, Sandhoff disorder; ERT, enzyme replacement therapy.

in patients with FD.[4,5] Nevertheless, ERT still presents important limitations, such as the low bioavailability and half-life of the drugs, the possibility to induce immunologic intolerance, and the incapability to cross the blood–brain barrier (BBB).[6]

Alternative therapeutic approaches, based on both in vivo or ex vivo gene therapy, are being tested to meliorate enzyme biodistribution and thus increase the therapeutic window for treatment administration and the possibilities of delivering into the central nervous system (CNS). The LSDs are particularly prone to be cured through gene therapy for the possibility of cross-correction of the enzyme defect,[7] and positive results have already been reported for metachromatic leukodystrophy.[8] Nevertheless, gene therapy still needs to be optimized for clinical applications, especially in terms of immunogenicity and control of viral genome integration sites.

A second alternative to ERT is represented by the use of orally administrated, no antigenic small molecules that can diffuse across multiple cell membranes and distribute in steady-state concentration through different tissues including the CNS. In this article, we will describe the characteristic of the different class of small molecules in use or in development for the treatment of LSDs.

Substrate Reduction Therapy

Small molecules can be used in LSDs to reduce accumulated substrate concentration by inhibiting the enzyme that synthesizes these substrates or one of their precursors. In this way, accumulation rate is lowered, facilitating the clearance of the storage by the defective, but yet partially functional, enzyme (Figure 1).[9]

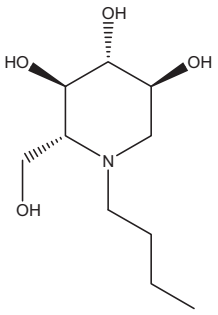
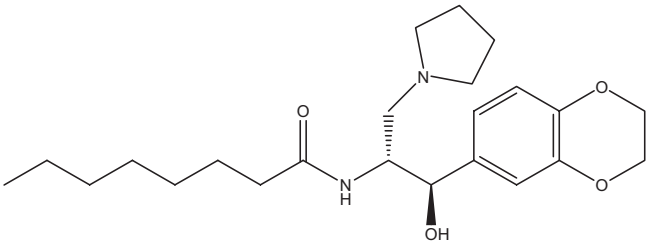
The SRT-based drugs are now available for the treatment of GD type I (Table 1), an LSD in which patients usually present with anemia, thrombocytopenia, hepatosplenomegaly, and skeletal deformations. However, in the most severe manifestations of the disease (GD type III), these symptoms are associated with neurological involvement. The GD is characterized by a deficit of β-glucosidase (also called glucocerebrosidase), which removes glucose moieties from glucosylceramide, causing the accumulation of the substrate, predominately in macrophages.[10]

Miglustat (*N*-butyldeoxynojirimycin), a competitive inhibitor of glucosylceramide synthetase, was the first SRT medicament to be approved with a therapeutic indication for patients with GD who cannot be treated by ERT (ie, for immunological reaction or low compliance).[11] Around 800 patients worldwide[9] currently use this drug, which is an oral agent with good gastrointestinal adsorption and wide tissue distribution, including the CNS. It seems that naive patients may respond later and with less efficiency to miglustat compared to ERT in terms of hematological and visceral values normalization[12]; however, patients who achieved therapeutic response through ERT may be switched to miglustat without compromising previous benefits.[13,14]

Miglustat causes nonsevere sides effects such as diarrhea, flatulence, abdominal pain, or weight loss, which are due to intestinal α-disaccharides malabsorption, probably related to the inhibition of α-glucosidase isomers.[15] Nevertheless, this effects can be minimized with continued treatment, dose escalation, and/or by implementing a low sucrose–maltose diet.[16] Tremor was also reported in 30% of the patients during trials and in postapproval treated patients,[17,18] since miglustat is a potent agonist of the human glucose sensor SGLT3, which is expressed at the neuromuscular junction.[19] However, tremor is generally mild and may be reverted with dose adjustment. Paresthesia and burning sensations were also reported, although it is not known whether they are drug dependent. The potential neurotoxicity of miglustat compromises its possible use in patients with GD type III with neurological involvement, who did not show any improvement during the clinical trials that were performed.[20]

Since miglustat can retard biosynthesis of glucosylceramide-based compounds, its efficacy was also tested for the treatment

Table 1. SRT for GD Type I.[a]

Properties	Miglustat	Eliglustat
Structure		
Targeted enzyme	Glucocerebrosidase	Glucocerebrosidase
Diffusion in CNS	Yes	No
Approved for other LSDs	NPC GD type I	GD type I
Comparison with ERT	Slower rate of substrate degradation	No inferior to imiglucerase
Administration form	Oral capsules	Oral capsule
Frequency	Three times per day	Extensive metabolizer: twice per day, poor metabolizer: once daily
Dose	100 mg	84 mg
Metabolization in human	No evidence	Yes (cytochrome P450)
Known interactions with drugs	Not known	Antiarrhythmics and other drugs metabolized by CP450
Agonist for	Glucose sensor SGLT3	P-glycoprotein transporter
Disaccharide absorption	Inhibits α-glucosidase isomers	No significant

Abbreviations: CNS, central nervous system; ERT, enzyme replacement therapy; GD, Gaucher disease; LSDs, lysosomal storage disorders; NPC, Niemann-Pick disease type C; SRT, substrate reduction therapy.
[a]Comparative description of the 2 small molecules used in clinical practice for GD type I treatment, which are based on SRT.

of Niemann-Pick disease type C (NPC), gangliosidosis, and MPS III (Sanfilippo syndrome).

The NPC1 and NPC2 are lipid storage disorders caused by mutations of genes involved in endosomal–lysosomal transport of lipids.[21] Cells, predominantly in the brain, accumulate unesterified cholesterol and other lipids with structural similarity to glycosphingolipids. Progressive accumulation determines that the disease manifestations may appear either at birth or later in life course, until the sixth decade of life. Onset in infants presents a more severe phenotype and may cause sudden failure, whereas late-onset patients develop neuropsychiatric manifestations and other neurological conditions, such as cerebellar ataxia, dystonia, dysarthria, seizure, cognitive problems, and progressive dementia.[22]

Treatment with miglustat managed to correct abnormal lipid trafficking in lymphocytes of patients with NPC-1 and to slow progression of neurological manifestations in animal models (mouse and cat) of the disease.[23,24] When used in clinical trial, miglustat improved saccadic eye movement and swallowing capacity of the patients, with better evidence in patients with later onset.[25,26] These results were confirmed in extended clinical trials, through assessments of cognitive functions and disability, and lead to the approval of miglustat for the treatment of NPC-1.[27]

Preclinical studies also showed positive results for the potential use of miglustat in the treatment of Tay-Sachs disorder (TSD) and Sandhoff disorder (SD). Both SD and TSD are gangliosidoses caused by the deficiency of β-hexosaminidase (β-Hex). β-Hexosaminidase is a dimeric enzyme (α and/or β subunits) that is required for the removal of the terminal *N*-acetylgalactosamine from several glycosphingolipids, as well as from some glycoproteins and oligosaccharides. Genetic mutations in the β-subunit result in compromised activity of both β-HexA and β-HexB leading to the accumulation of glycosphingolipids, GAGs, acetylgalactosamine, and *N*-acetylglucosamine, which are responsible for SD. On the other end, TSD results from mutations in the α-subunit, leading to β-HexA deficiency and the accumulation of ganglioside GM-2 as major storage product.[28] In TSD, GM-2 accumulation is confined to CNS, whereas in SD, there are multisystemic manifestations.

In mouse model of SD, miglustat was able to reverse neuronal pathophysiological and biochemical abnormalities, as well as to delay symptom of onset and to increase life expectancy. Moreover, treatment with the same drug prevented accumulation of GM-2 in the brain of the TSD model.[29,30]

Unfortunately, these results were not confirmed in a trial performed in 5 teenagers with GM-2 gangliosidosis observed over 2 years, failing to prevent neurological deterioration,[31] nor it did with patients with TSD,[32] in spite of previously reported positive data of an isolated study performed with patients with SD.[33]

Treatment with miglustat also failed to show any improvement or stabilization in patients with Sanfilippo syndrome, either in terms of clinical condition or of ganglioside concentration in the brain.[34] Genistain, another SRT-based compound, was also reported to improve cognitive and behavioral

functions in MPS III,[35] although a more extended trial did not confirm the initial results.[36]

The second SRT-based treatment for GD type I to be approved was eliglustat, a highly specific oral glucosylceramide synthase inhibitor structurally different from miglustat.[37] In a double-blind 9-month phase III multinational clinical trial (ENGAGE, Phase III, randomized double-blind, placebo controlled, multicentre study confirming the efficacy and safety of Genz-112638 in patients with Gaucher disease Type I, NCT00891202), the drug significantly improved hematological parameters, bone marrow burden score, and also reduced organomegaly in naive patients with GD type I.[38] Moreover, in a second clinical trial, comparing eliglustat to ERT (a study of Eliglustat tartrate (Genz-112638) in patients with Gaucher disease, who have reached therapeutic goals with enzyme replacement therapy, NCT00943111), the end points that were reached proved that this drug is not inferior to imiglucerase after 12 months of treatment.[39] Eliglustat does not inhibit intestinal glucosidases and had no neurotoxic effect, since it is a substrate for P-glycoprotein transporter and is extruded from the CNS.[40] On the other end, other P-glycoprotein substrates such as digoxin, phenytoin, and colchicine can compete with eliglustat, affecting its pharmacokinetics. This active compound is metabolized through the cytochrome P450 complex, therefore, it can potentially interact with other drugs that are degraded through the same pathway. Pharmacokinetics of eliglustat can also be affected by the genetic heterogeneity of the patients with respect to the CYP2D6 enzyme of the cytochrome P450 complex. Prior to initiating eliglustat therapy, patients should be genotyped for CYP2D6 to determine their CYP2D6 metabolizer status and to find the adequate individual dosage. For this reason, the medicament obtained therapeutic indication for patients with GD type I, who are CYP2D6 extensive metabolizers, intermediate metabolizers, or poor metabolizers.[41,42]

The SRT is also used for the treatment of infantile nephropathic cystinosis, a rare genetic disease due to dysfunction of cystinosine transporter expressed in lysosomal membrane. Cysteamine converts accumulated cystine into cysteine and cysteine–cysteamine mixed disulfide, both of which can pass through the lysosomal membrane of patients with cystinosis.[43] Cysteamine bitartrate has been approved by the Food and Drug Administration since 1994, and in 2013, a delayed release formulation, no inferior to the previous one, was also approved.[44,45] In spite of its use in clinical practice, long-term studies indicate that cysteamine treatment even when started early (before 5) is not curative and 30% of the patients require renal transplant before 16 years.[46] The same active compound was also tested for the treatment of neuronal ceroid lipofuscinosis caused by *PPT1*, which is involved in lysosomal degradation of S-fatty acylated proteins.[47]

Pharmacological Chaperones

In several LSDs, substrate accumulation that determines clinical manifestations occurs when residual enzyme activity decays below a certain threshold. It was determined that an activity >10% significantly prevents storage in many LSDs and even values between 3% and 5% could be sufficient to slow down the disease progression.[48,49] In terms of treatment, this means that even a low improvement in enzyme activity could be effective to meliorate clinical evidence. A pharmacological chaperone (PC) is a small molecule that stabilizes the tertiary structure of a mutant enzyme, avoiding its degradation by the quality control mechanisms of the endoplasmic reticulum (ER) and allowing its trafficking to the lysosome through the secretory pathway (Figure 2).[50] Once the complex has reached the target organelle, the PC is released from the enzyme upon acid hydrolysis and the protein can, at least partially, catalyze substrate degradation, still in the presence of the mutation. Even though lysosomal enzymes are active at acid pH, their synthesis normally occurs at neutral pH; therefore, these proteins may be thermodynamically less stable during synthesis process, especially in the presence of a mutation. The PCs stabilize protein conformation, inhibit premature misfolding, and prevent aggregation.

Several PCs were designed and synthesized based on the structure of the natural substrate (iminosugars, azasugars, carbasugars, etc) or alternatively identified by high-throughput screening (HTS) studies. These compounds, potentially active for LSDs therapy, showed encouraging results in preclinical studies for the treatment of FD, GD, PD, or gangliosidosis 1 and 2 (Table 2).

In 2016, the first PC-based therapy (Galafold, Amicus Therapeutics) obtained marketing approval by the European Medicines Agency for the treatment of patients with FD with specific missense mutations. The active compound of this drug is 1-deoxygalactonojirimycin (DGJ), an iminosugar mimicking the structure of D-galactose, which is also denominated migalastat.[57] The FD is an X-linked LSD, caused by the impairment of α-galactosidase A (α-GalA), which leads to the progressive accumulation of glycosphingolipids such as globotriaosylceramide (GL-3) and Lyso-GL3 in vascular endothelium, smooth muscle, kidney, dorsal ganglia, brain, gastrointestinal tract, and so on.[58]

The chaperoning action of DGJ depends on the interaction of its amino group with the carboxylate of the aspartic acid 170 in the active site of the enzyme, as shown by crystallography.[59] Exposure to DGJ (0.2-20 μmol/L) of primary lymphoblast cultures from patients with FD caused a concentration-dependent increase in α-Gal A activity, which was maintained for 5 days.[57] In vivo studies with DGJ on FD transgenic mouse models showed significant increase in α-Gal A activity in heart, kidney, spleen, and liver of the treated animals, as well as decreased levels of GL-3 in the affected tissues.[59] A phase 2 clinical trial performed with migalastat hydrochloride as monotherapy showed increased α-Gal A activity and decreased GL-3 concentration in skin, urine, and kidneys. Reduction in renal peritubular capillary inclusions was also found in heterozygous women.[60] In phase 3 studies, stabilization of renal function, reduction in left ventricular mass, and improvement in gastrointestinal symptoms were demonstrated, as well as a generally good tolerability.[61] Long-term effects of the drugs as well as its possible synergistic effect in the cotreatment with ERT are currently being evaluated.

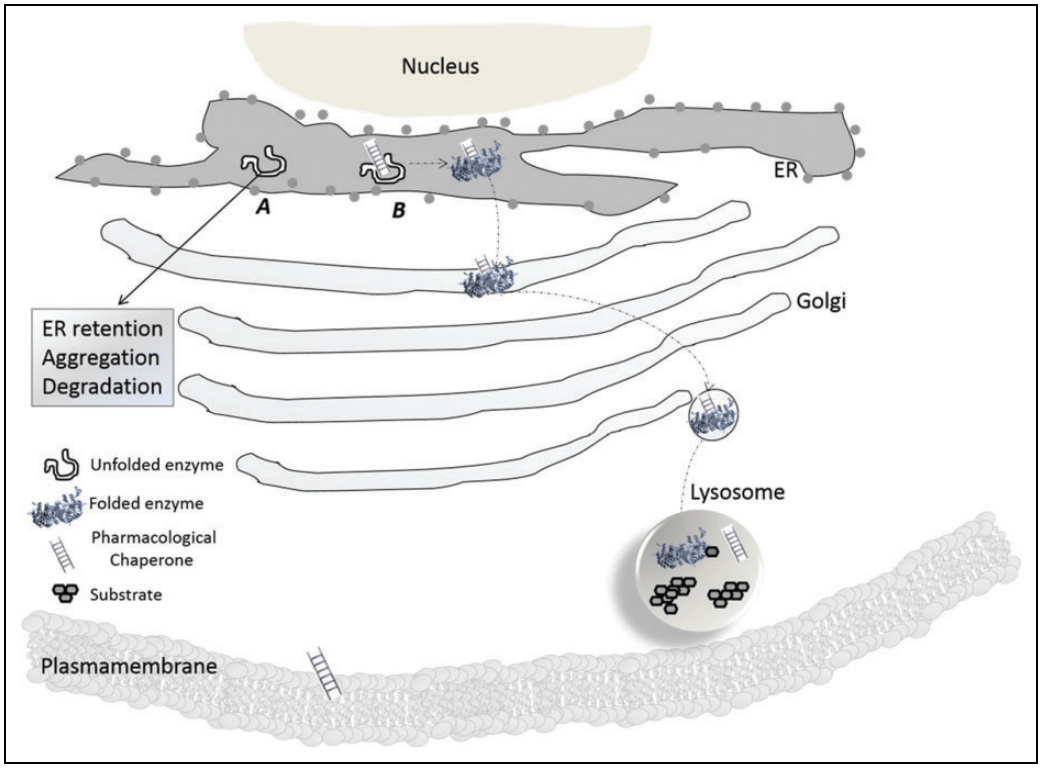


Figure 2. Pharmacological chaperones: mechanism of action. A, Mutated lysosomal enzyme that does not fold correctly is degraded at ER level. B, Pharmacological chaperone diffuses through the plasma membrane into the cell and binds to the mutated enzyme, allowing proper folding of the protein. The complex traffics through the secretory pathway and enters the lysosome, where the PC is released and the enzyme can catalyze substrate degradation. ER indicates endoplasmic reticulum; PC, pharmacological chaperone.

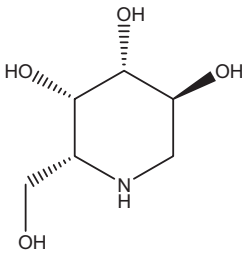
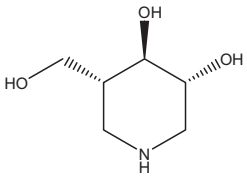
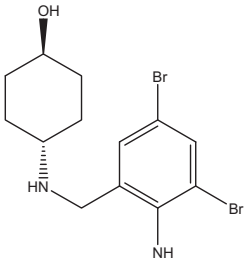
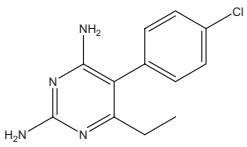
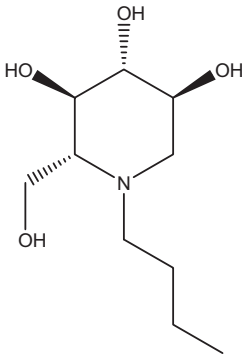
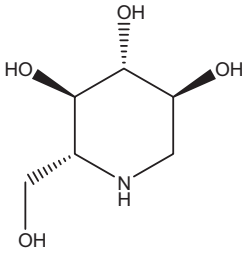
Two potential PCs for GD, isofagamin and ambroxol, were identified through HTS.[62,63] Isofagamin increased β-glucosidase activity in cells derived from p.N370S- and p.L444P-affected patients.[64] These results were confirmed in transgenic mice with different mutations including the p.L444P[65,66] and put the basis for phase 1 and 2 clinical trials, which unfortunately were discontinued, failing to show a clinically meaningful end point in most of the involved patients (NCT00446550 and NTC00875160). Ambroxol was also able to stabilize p.N370S-mutated β-glucosidase in patient's derived cells and animal models.[67,68] In clinical trials, ambroxol showed reduction of 15% to 40% in spleen volume and increased platelet counts after more than 6 months therapy in 1 patient.[53]

The PCs have been proposed to complement ERT also for PD. The PD is an LSD caused due to mutations in *GAA* gene, which encodes acid α-glucosidase. The enzymatic deficit produces accumulation of glycogen predominantly in heart and skeletal muscles.[69] Both 1-deoxynojirimycin (DNJ, duvoglustat) and miglustat can increase acid α-glucosidase activity in cells expressing different mutant forms of the enzyme and in animal models, since these iminosugars are 10- to 250-fold more selective for α-glucosidase compared to β-glucosidase.[70,71] In particular, DNJ administration to PD mouse model showed daily important reduction of substrate in heart, diaphragm, gastrocnemius, soleus, and brain.[72] In spite of the positive tolerability results of phase 1 studies, duvoglustat showed severe adverse events in 2 patients during phase 2 clinical trial.[55] A follow-up study, showing the pharmacokinetic profile of the drug in the muscle, demonstrated that the dose selected for phase 2 study was excessive (>Half Maximal Inhibitory Concentration $[IC]_{50}$ for inhibition of the enzyme), and therefore, appropriate balance between chaperoning and inhibition was not achieved, causing the adverse events.[56]

On the other end, miglustat was tested as a combination therapy for PD to enhance the effects of ERT in patients with infantile and late onsets in a phase 2 trial. In the whole population (13 patients), acid α-glucosidase activity was significantly increased with the cotreatment.[73]

Since PCs can diffuse to the CNS, they are a potential good treatment for LSDs with important neurological manifestation such as SD and TSD, since ERT drugs do not cross the BBB. Among the tested compounds for SD and TSD, pyrimethamine, an approved antimalarial agent, significantly increased enzymatic activity in all the β-subunit mutants evaluated; however, it determined an insufficient increase in enzymatic activity of the α-subunit mutants.[54] In spite of this limitation, a clinical trial phase 1/2 was initiated in patients with SD or TSD. The 8 treated patients showed significant enzymatic activity increase; however, severe side effects, such as ataxia or blurred vision, among others, appeared during the trial, causing the suspension of the study.[74] Likewise, in the case of duvoglustat trial in

Table 2. Clinical Trials of PCs.[a]

Compound	Structure	Diseases	Clinical Trial	Phase	References
Migalastat		FD	NCT01458119	Approved	[51]
		FD (long-term study)	NCT02194985		
Migalastat + ERT		FD	NCT01196871	II	[52]
Isofagomine		GD	NCT00875160	II	
Ambroxol		GD	NCT01463215	I/II	[53]
Pyrimethamine		SD and TSD	NCT01102686	I/II	[54]
Miglustat + ERT		PD	NCT02185651	I	[55]
Duvoglustat		PD	NTC00688597	II	[56]
Duvoglustat + ERT		PD	NCT01380743	II	

Abbreviations: ERT, enzyme replacement therapy; FD, Fabry disease; GD, Gaucher disease; PCs, pharmacological chaperones; SD, Sandhoff disorder; TSD, Tay-Sachs disorder; PD, Pompe disease.

[a]PC has been or is being tested in clinical trials, either in monotherapy or in combination with ERT. The identification number of the clinical trials corresponds to the database clinicaltrial.gov (https://clinicaltrials.gov).

patients with PD, it is possible that the dose of pyrimethamine selected for the study was too elevated for appropriate balance between chaperoning and inhibitory activity of the molecule. The majority of PCs that have been proposed binds to the active center of the target enzyme, therefore, they may act as inhibitors if the affinity is too high. This represents a major limitation for PC therapies for which the concentration has to be finely tuned to determine a net gain in chaperoning activity. Turnover is favored by high substrate concentration and by binders that present shorter half-life than the enzyme, as well as reduced binding affinity at lysosomal pH.

The increase in activity, however, depends on the disease and on the affected enzyme maturation process and its lysosomal trafficking. Moreover, the efficacy of PC-based drugs is mutation dependent, and a single compound may have different effects on patients with the same LSD, but different mutations. The PCs are more prone to stabilize proteins with missense mutations (either inside or outside the active site), which affect protein folding, thermodynamic stability, or lysosomal trafficking, while they are not suitable for large deletions, insertions, splice variants, or frameshift mutations.

Stop-Codon Read-Through Enhancement

Many patients with LSD present nonsense mutations in one or both alleles of the gene encoding for the lysosomal enzyme, which generate a premature stop codon. As an example, mucopolysaccharidosis type I (Hurler and Scheie syndromes), an LSD caused by the deficit of α-L-iduronidase, is often due to the frequent mutations p.Q70X and p.W402X in the *IDUA* gene.[75] In this disease, as well as in neuronal ceroid lipofuscinosis type I, over 50% of the patients carry a nonsense mutation.[76,77] Premature stop-codon mutations are usually associated with more severe clinical phenotypes, since truncated messenger RNA (mRNA) or protein is generally eliminated with resulting null or minimal net enzyme activity in the cells. Most mRNA transcripts containing stop-codon mutations are preferentially targeted for degradation by a cellular quality control system called nonsense-mediated decay, which limits the synthesis of potentially toxic or unwanted protein fragments that might interfere with normal cellular function.[78] Normally, during the splicing process, an exon–exon junction complex is recruited to mRNA and binds 20 to 24 nucleotides upstream of the exon–exon junction. A premature stop codon, upstream of this junction can elicit nonsense-mediated decay.[79] If a protein escapes this degradation mechanism, a truncated polypeptide is produced and often degraded by the control mechanisms of the ER. However, the read-through process may originate normal or mutated full-length proteins by incorporating an amino acid instead of terminating the synthesis at the premature stop codon. These products can be enough stable to bypass the ER degradation system and perform its function within the cell (Figure 3).

The read-through process can also be induced by drugs, such as gentamicin or other aminoglycosides, which bind to ribosomal RNA and affect the stop-codon transfer RNA recognition process.[80,81] The degree of read-through process will depend on the amounts of

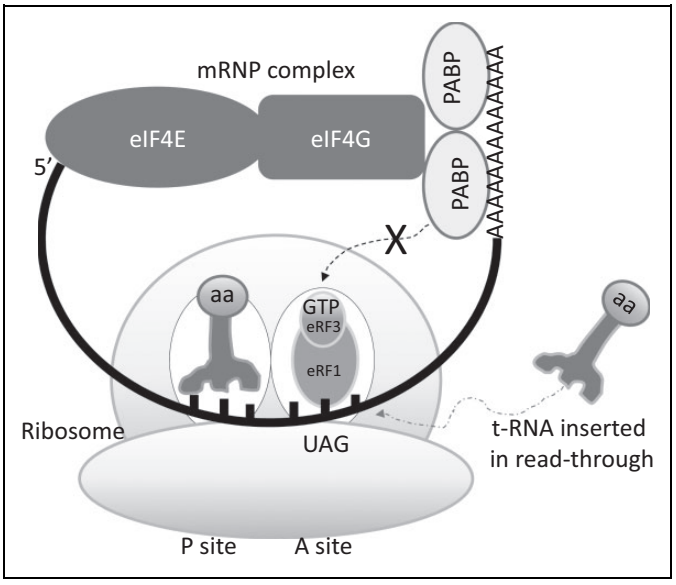


Figure 3. Stop-codon read-through process. The association of 3 proteins at the messenger ribonucleoprotein (mRNP) complex leads to a circular messenger RNA (mRNA) structure. The cap-binding protein eukaryotic initiation factor 4E (eIF4E) binds the 5′ extremity of mRNA; the poly(A)-binding protein (PABP) binds the 3′ end of mRNA; and the eIF4G protein binds both eIF4E and PABP. In mRNAs with wild-type stop codons, PABP is close to the termination complex and can interact with termination factor eukaryotic release factor 3 (eRF3) to stimulate polypeptide chain release. In the presence of a premature stop codon, PABP is at a larger distance and PABP interaction with eRF3 is more difficult, which increases the possibility of activate aminoacyl-transfer RNA to reach the complex and follow the translation (read-through process). Termination factor eRF1 directly recognize any of the 3 stop codons.

available RNA, the kind of stop codon of the nonsense mutation (UAA has high fidelity and little read-through potential, the UAG has intermediate fidelity, and the UGA sequence has the lowest fidelity and higher potential), the surrounding sequence (ie, nucleotide plus 4 is determinant), and correlates with the efficacy of nonsense-mediated decay.

The amino acid that is inserted in read-through process is not always the correct one, but the protein that is produced may still be stable. The amino acids that are inserted with the highest probability during read through are the glutamine and the tryptophan, which is an advantage in the case of patients with MPS I carrying p.Q70X or p.W402X mutations.[82] Gentamicin gave promising results when tested in vitro for cystic fibrosis or Duchenne muscular dystrophy and also for MPS I, among the LSDs.[81,83,84]

The principal limitations for the use of gentamicin as read-through inducer are its low cell permeability and its toxic side effects, including kidney damage and hearing loss. For this reason, different analogues of this compound have been proposed. It has been shown that the C1a-subunit of gentamicin (amikacin) preserves efficacy but has reduced side effects.[85] Another stop-codon read-through active compound, which was identified during HTS, is PTC124. This active molecule, which inhibits nonsense-mediated decay and allows read through

without affecting normal termination codons, presents high permeability and no toxic side effects.[86]

PTC124 proved to be more efficient than gentamicin in cystic fibrosis and Duchenne muscular dystrophy in preclinical studies. Phase 3 clinical trials were performed in patients with cystic fibrosis and Duchenne muscular dystrophy (NCT00803205 and NCT0182647), leading to the conditional approval of the compound for Duchenne muscular dystrophy.[87,88]

PTC124 was also tested in cells derived from patients with MPS VI (Maroteaux-Lamy). The MPS VI is an LSD without CNS involvement, which is caused by arylsulfatase B (*ARSB*, gene) deficiency. It is characterized by dysostosis multiplex, corneal clouding, heart valve defects, and urinary excretion of GAGs. A subgroup of patients with MPS VI carries nonsense mutations (p.R315X, p.R327X, p.Q456X, and p.Q503X) and therefore is eligible for enhancing of stop-codon read through, using small molecules. PTC124, but not gentamicin, showed to significantly increase the level of arylsulfatase B activity, resulting in a significant reduction in lysosomal size.[89]

In a second study, gentamicin, geneticin, PTC124, and 4 no aminoglycosidic compounds (RTC13, RTC14, BZ6, and Bz16) were tested in fibroblast derived from patients with MPS VI and MPS III B or C (Sanfilippo) with premature stop-codon mutations. Cells from 1 patient with Maroteaux-Lamy carrying the p.W322X mutation responded to treatment with gentamicin (2- to 3-fold increase in enzyme activity); however, the effects of the other compounds were not tested.

In Sanfilippo B and C patients' cells, an increase in mRNA levels of *NAGLU* and *HGSNAT* was found after treatment with, respectively, geneticin and RTC14 or PTC124, although no enzyme activity was recovered.[90]

Conclusions and Future Perspectives

The use of small molecules, comprising SRT, PCs, and read-through enhancer, could represent a great advantage in the treatment of LSDs compared to ERT, since they can be administrated orally, achieve homogenous steady-state concentrations, and possibly lower production costs. Nevertheless, important challenges have still to be faced to advance in the development of these drugs.

Small molecules can potentially diffuse in the CNS; however, up to date, the only commercialized treatment for LSDs with neurological involvement is miglustat, which has a therapeutic indication for NPC, and there is still no available treatment for a considerable number of LSDs. Indeed, the clinical trials performed in GD type III with miglustat or in gangliosidoses with pyrimethamine did not give the expected results due to toxic side effects, which were probably related to unoptimized doses.

Reviewing patents databases[6] (https://worldwide.espacenet.com/), many registered molecules claim PC activity for LSDs, nevertheless, at the moment the only PC to be commercialized is migalastat. The delay in the translation to the clinic could be related to the difficulty to select an appropriate dose of administration for PC-based treatment, which should allow maximal substrate turnover without inhibitory effect. An option to reach this goal is to administer the PC in alternative intervals, since the turnover of the small molecule is generally faster than that of the defective enzyme. In hR301Q α-GalA transgenic mice, oral administration of migalastat in cycle of 4 days during a week resulted in greater substrate clearance, compared to daily administration.[90]

Alternatively, next-generation PC should be directed to the domains of the lysosomal enzyme, which do not include the active site but have a crucial role in the stabilization of the protein (allosteric sites). This will preserve PC scaffolding function without affecting its catalytic action. Potential PCs binding to allosteric sites have already been identified for acid α-glucosidase[52,91] and glucocerebrosidase.[92,93]

Finally, a therapeutic option that is being explored for the treatment of LSDs is the coadministration of ERT with small molecules. Among the limitations of ERT, low half-life and uneven distribution of the recombinant enzyme are crucial in lowering the efficacy of the treatment. As an example, ERT for PD has to be administered at higher frequency and dose compared to other enzyme, due to the low expression of MP6 receptors in the muscle and the higher adaptive immune system activation in these patients. Nevertheless, it has been shown that wild-type lysosomal enzymes like the ones affected in FD, PD, and GD respond to PCs, increasing the rate of substrate degradation. Interestingly, only some of the wild-type enzymes related to LSDs respond to chaperons, depending on the folding efficiency of the enzyme, which may also be different across cell types and species. As mentioned previously, cotreatment was tested in patients with PD, achieving significant increase in blood α-glucosidase activity by administering miglustat or eliglustat prior to ERT.[55,56] Similar results were obtained in patients with FD treated with migalastat before ERT infusion, increasing α-Gal A activity in the blood and the skin of all the patients.[94,95]

For their action mechanism, SRT and read-through enhancer can also potentially contribute to higher storage metabolization, when used in combination with ERT. Therefore, cotreatment could be the key to achieve clinical improvement in patients with LSDs.

Due to the heterogeneity of LSDs and the variability of the expressed phenotypes, the ideal treatment for all affected patients has not been developed up to date. Nevertheless, thanks to the recent advances exposed in this review, crucial problems and possible solutions have been identified and will allow to design new tools and specific management protocols based on the association of different therapeutic agents to face individual diversity.

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