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Combination therapies for lysosomal storage disease: is the whole greater than the sum of its parts?

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Lysosomal storage diseases (LSDs), as a group, are among the most common inherited diseases affecting children. The primary defect is typically a genetic deficiency of one of the lysosomal enzymes, often causing accumulation of undegraded substrates within the lysosome. This accumulation causes numerous secondary effects that contribute to the disease phenotype. Viral-mediated gene therapy (GT) can supply a persistent source of the deficient enzyme. However, with some notable exceptions, GT has been only modestly successful as a single approach. Recently, various therapies have been combined in order to more effectively target the diverse pathogenic mechanisms at work in LSDs. One strategy that has shown promise involves providing a persistent source of the deficient enzyme (GT, stem cell transplantation) while targeting a secondary consequence of disease with a more transient approach (substrate reduction, anti-inflammatories, pharmacological mimetic, etc.). This general strategy has resulted in both additive and synergistic effects. Interestingly, some therapeutic approaches by themselves provide essentially no clinical benefit but contribute greatly to the overall efficacy when used in combination with other treatments. Unfortunately, no therapeutic combination is universally effective. This adds to the difficulty in predicting and identifying combinations that will be most effective for individual LSDs. A better understanding of both pathogenic and therapeutic mechanisms is necessary in order to identify potentially successful combinations. While a single treatment would be ideal, the complex nature of these diseases may unavoidably limit the efficacy of single therapies. In order to more successfully treat LSDs, a shift in focus towards a combination therapy may be necessary.

INTRODUCTION

Lysosomal storage diseases (LSDs) are a class of 50 or more inborn errors of metabolism, which typically result from a defect in a soluble lysosomal enzyme (1). However, several LSDs are caused by defects in integral lysosomal membrane proteins. While individual LSDs are rare, when considered as group they are one of the most common genetic disorders in children, affecting ~1 out of every 7000–8000 live births (2–4). Lysosomes from affected individuals become distended; engorged with substrates normally degraded by the deficient enzyme. This catabolic block can lead to numerous secondary changes, which contribute to the complex nature of these diseases. These secondary effects include oxidative stress (5,6), endoplasmic reticulum (ER) stress (7,8), defects in autophagy (9), altered calcium homeostasis (10–12) and a significant energy imbalance (13,14). Most lysosomal enzymes are ubiquitously expressed; therefore, disease often affects multiple organ systems. The clinical signs of LSDs can vary, but often include hepatosplenomegaly, cardiac disease, abnormal skeletal growth and immune defects (1,15,16). The brain is particularly sensitive to the loss of these enzymes, with ~75% of LSDs having significant central nervous system (CNS) involvement. Clinical signs associated with CNS disease include cognitive delays, auditory and visual defects, seizures, and peripheral neuropathy (1).

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CURRENT AND FUTURE THERAPIES FOR LSDs

A number of therapeutic approaches are currently being developed for LSDs. The vast majority of treatments are targeted towards defects in soluble lysosomal enzymes, as there is a conceptual framework for the replacement of these enzymes. Reconstituting soluble enzyme deficiencies can be accomplished either directly through enzyme replacement therapy (ERT) or indirectly through bone marrow transplantation or GT. ERT is conceptually the most straightforward, relatively non-invasive and directly targets the primary enzyme deficiency. ERT is available for a small number of LSDs, including Gaucher, Fabry, MPS-I, MPS-VI and Pompe disease. Owing to the transient nature of ERT, it is typically administered weekly, which requires a significant investment of time and resources. In addition, lysosomal enzymes generally do not cross the blood–brain barrier (BBB), limiting the value of ERT for LSDs with significant CNS involvement. Hematopoetic stem cell transplantation (HSCT) using either bone marrow or cord blood has also been utilized as a treatment for many LSDs. The goal of HSCT is to provide a widespread and continuous source of the deficient enzyme from hematopoetic-derived cells. However, significant risks are associated with this approach, including harsh conditioning regimens and graft-versus-host disease. For this reason, HSCT is generally limited to LSDs that show a clear beneficial response and for which ERT is not available (17–20). While ERT and HSCT can prolong and improve the quality of life for patients with some LSDs, neither of these treatments is curative.

LSDs are particularly good targets for GT for several reasons. First, LSDs are single-gene defects. Therefore, providing the correct gene for the disease is conceptually straightforward. Second, there is little concern about the inadvertent genetic modification of an inappropriate cell type as lysosomal enzymes are ubiquitously expressed. Third, precise transcriptional regulation is probably not necessary as over-expression of lysosomal enzymes does not appear to be detrimental and as little as 5–10% normal levels of enzyme can be therapeutic. Finally, because soluble lysosomal enzymes can 'cross-correct' their neighboring cells (21,22), the number of cells that must be modified with a gene transfer vector is relatively low.

Recombinant viral gene transfer vectors are currently the most effective means of gene transfer and enzyme expression. Multiple viral vectors have been utilized for the treatment of LSDs. These include γ-retroviruses, adenovirus (Ad), herpes simplex virus (HSV), adeno-associated virus (AAV) and lentiviruses (lenti), to name a few. Adenoviruses can very efficiently infect non-dividing cells *in vitro* and *in vivo* (23). However, the profound immunogenic nature of the early generation vectors has limited their utility (24,25). The γ-retroviruses have been used primarily in *ex vivo* applications, where progenitor cells (typically hematopoietic) are transduced in culture and transplanted into a recipient (26–28). The γ-retroviruses infect only the dividing cells. Therefore, with several notable exceptions, their direct *in vivo* applicability is limited as many GT target organs consist primarily of non-dividing cells. In contrast, lentiviruses can infect both dividing and non-dividing cells, and are appropriate for both *ex vivo* (29,30) and *in vivo* (31–33) applications. Adeno-associated viral vectors effectively transduce many cell types *in vivo*. In addition, AAV vectors can be pseudotyped with capsid proteins from different AAV serotypes, which dramatically alter their tropism (34). Owing to this and their low immunogenicity, AAV and lentiviral vectors are widely used for systemic- and CNS-directed GT. Specific viruses or serotypes may be more appropriate for certain applications. For example, primarily neurodegenerative LSDs such as Krabbe Disease, Infantile Neuronal Ceroid Lipofuscinoses (NCL) and Sandhoff Disease, just to name a few, may require the use of viral vectors with neuronal or glial tropisms. The identification of new AAV serotypes, each possessing their own unique host cell affinity, is ongoing and promising. HSVs have also been pseudotyped to improve transduction specificity in the CNS of mice (35). For a more comprehensive list of viral vectors and their applications for LSDs see Sands and Davidson (36).

The route of viral vector delivery can vary greatly. The chosen method of administration depends on several factors, including ease of vector delivery and the site(s) of most significant disease. An effective strategy to treat the systemic disease associated with LSDs is to create an 'enzyme factory organ', which would secrete high levels of enzyme, resulting in persistent circulating enzyme available to visceral tissues. This was first accomplished in animal models via *ex vivo* transduction of hematopoetic stem cells in MPS-VII (28), Fabry Disease (37), GM1 gangliosidosis (38) and Neiman-Pick A/B (27). The goal was to generate an enzyme delivery vehicle (hematopoietic-derived cells) that had access to most tissues and utilized the patients own cells to minimize graft-versus-host disease. While theoretically promising, this approach is complicated, requires harsh conditioning regimens and provides limited enzyme delivery. In a related approach, human bone marrow-derived mesenchymal stem cells were transduced *ex vivo* with a lentiviral vector expressing β-glucuronidase and transplanted intraperitoneally into the xenotransplant model of MPS-VII (NOD/SCID/MPS-VII) (39,40).The transduced cells migrated into many tissues, persisted for the length of the study (4 months), expressed high levels of enzyme and reduced lysosomal storage in several critical tissues. Unfortunately, little or no enzyme was detected in the CNS and there was no reduction of storage material in the brain. Intramuscular delivery of GT has also been attempted in LSDs, as transduced myoblasts were shown to secrete lysosomal enzymes *in vitro* (41–43). However, fully differentiated myofibers secrete very little enzyme *in vivo*, which reduces the efficacy of this approach (44–46).

Considerable success has been achieved with the intravenous (i.v.) delivery of GT vectors, which is relatively non-invasive. This method has the advantage of widespread vector delivery, although much of the dose is generally sequestered by the liver (31,47). Interestingly, efficient transduction of the liver has benefits. It has been shown that the liver secretes more enzyme than other tissues (47–49), making the liver an ideal 'enzyme factory organ'. Intravenous administration of gene transfer vectors has been rather successful in some of the MPS disorders and Fabry disease utilizing Ad (50), AAV (44,51–53) and lentiviral (31,32) vectors. Direct intrahepatic administration of an AAV2 vector in the

MPS-VII mouse has also shown some promise (54), however this approach is rather invasive. Preclinical animal data suggest that MPS-I (52) and MPS-VII (47,49,51) may be quite effectively treated by a single i.v. dose of an Ad, AAV or retroviral vector delivered during the neonatal period. Other models studied, however, have shown only modest improvements in lifespan, behavior or molecular markers of disease following single-dose GT (55,56).

Although effective in treating visceral pathology, systemic delivery of GT can generally provide only minimal benefit to the CNS owing to the BBB. The BBB effectively excludes most peripherally administered gene transfer vectors and enzymes. As many LSDs have a significant neurological component, this is a major limitation to systemic delivery. However, viral gene transfer vectors can be administered directly into the CNS. Direct CNS delivery has two primary advantages: (1) the gene transfer vector and protein expression is delivered directly to the site of disease, and (2) systemic dissemination of vector is limited, and as the CNS is considered an 'immune-privileged' site, the immune response to the vector may be less robust. Direct CNS delivery has taken many forms including: intraparenchymal (31,33,57–62), intraventricular (63) and intrathecal (64,65) injections.

The primary disadvantages of direct CNS delivery are the invasive nature of the procedure and the limited diffusion of the vector within the brain. However, CNS-directed GT can be augmented through axonal transport (66,67), whereby vector and/or enzyme administered at a remote location (e.g. eye) is transported into the CNS. Axonal transport can also be utilized to increase the distribution of the enzyme within the CNS (68,69). It was shown that transduction of the ventral tegmental area in the mouse, which has axonal connections to many areas of the brain, results in enzyme distribution throughout the neuroaxis (69,70). Administration of viral GT has been performed in the CNS of children with late infantile NCL (70,71). While some complications were observed, this approach was generally well tolerated, thus paving the way for future therapies utilizing CNS delivery of GT for LSDs.

It may also be possible to develop vectors and delivery strategies that allow GT across the BBB. Recent preclinical work has shown promise by targeting a modified AAV vector to the brain endothelium, which can be transduced through an i.v. injection. The transduced endothelium provides enzyme to much of the brain, presumably through basolateral secretion (63). Additionally, systemic GT could be administered at birth, before the BBB is intact; however, this is difficult without newborn screening programs, which are not in widespread use. Lastly, evidence is building that a newer AAV serotype, AAV9, can effectively cross an intact BBB and transduce various cell types in the brain. This viral vector may prove particularly useful for the CNS delivery of GT.

COMBINATION THERAPIES FOR LSDs

Promising therapeutic approaches for LSDs are in development. However, there are only isolated instances where a single approach corrects most of the biochemical, histological and clinical features of the disease. This is likely owing to the complex nature of these diseases, as well as the inaccessibility of certain tissues, primarily the CNS. In addition, most of the promising results have been observed under carefully controlled laboratory conditions. Although the primary insult in LSDs is a single-gene defect, multiple secondary mechanisms play a role in the pathogenesis (72). These include accumulation of secondary metabolites, altered calcium homeostasis, oxidative stress, inflammation, abnormal lipid trafficking, increased autophagy, ER stress, unfolded protein response and autoimmunity. Each of these secondary effects is a potential therapeutic target (Fig. 1). It is likely that one or more of these pathogenic mechanisms are at play by the time therapy is initiated, limiting the efficacy of the primary approach. Therefore, several groups have begun combining therapies in order to target either different aspects of disease or different tissues. Additionally, the timing of various therapies can, and has been optimized in order to take advantage of the strengths of each approach. Interestingly, both additive and synergistic effects have been documented when two or more treatments are combined (Table 1).

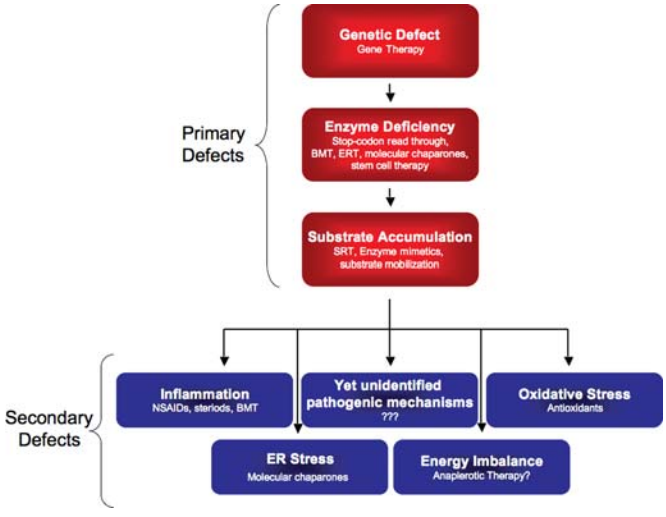

Figure 1. Pathogenic mechanisms involved in LSDs. The disease mechanisms can be divided into primary (red) and secondary (blue) defects. A few or nearly all of these mechanisms may be present, depending on the specific LSD. Each defect represents a possible therapeutic target. By treating more than one aspect of the disease, additive or even synergistic efficacy can be achieved.

In one of the first examples of combination therapy, weekly ERT beginning at birth was followed by bone marrow transplant (BMT) at 5 weeks of age in the murine model of MPS-VII (73). The goal was to provide an immediate source of enzyme through ERT while delaying BMT until the deleterious effects of harsh conditioning during the newborn period could be minimized. Although each therapy alone was relatively effective when initiated early in life, the addition of BMT to ERT resulted in a more widespread enzyme distribution and reduced lysosomal storage in the bone, meninges, cornea and retina. In addition, the radiation-induced damage observed when BMT is performed in the neonatal period was avoided.

Substrate reduction therapy (SRT) has been utilized in several LSDs. The goal of SRT is to reduce the accumulation of metabolites by inhibiting their synthesis. As the synthesis of

Table 1. Pre-clinical combination therapies for LSDs

Disease	Treatment	Efficacy	Reference
MPS-VII	ERT and delayed BMT	[a]	(73)
MPS-IIIB	BMT and CNS-directed GT	[b]/[c]	(82)
Sandhoff	BMT and SRT	[d]	(76)
	SRT and aspirin	[d]	(77)
	SRT and neuronal stem cell therapy	[d]	(78)
	SRT and anti-inflammatory or antioxidant therapy	[a]	(77)
Globoid cell leukodystrophy	BMT and SRT	[d]	(81)
	BMT and CNS-directed GT	[d]	(56)
	BMT and antioxidant therapy	[b]	
INCL	BMT and CNS-directed GT	[d]	
	BMT and enzyme mimetic therapy	[c]	
Niemann-Pick A	CNS-directed and systemic GT	[d]	(79)

[a]Additive improvement in lifespan as well as behavioral or biochemical markers of disease.
[b]No improvement in any markers of disease.
[c]Modest improvement in behavioral or biochemical markers of disease, but no improvement in lifespan.
[d]Synergistic improvement in lifespan as well as behavioral or biochemical markers of disease.

substrates cannot generally be shut down completely, this approach alone will likely only slow the inevitable progression of disease. In the murine model of Sandhoff disease (β-hexosaminidase A deficiency), SRT can be achieved with *N*-butyldeoxynojirimycin (miglustat), which inhibits the first committed step in glycosphingolipid synthesis (74,75). This effectively reduces the accumulation of total brain ganglioside and GM2 in the mouse (74). Combining SRT with BMT resulted in a synergistic improvement in lifespan (76). In the same model, SRT was combined with anti-inflammatory therapy (indomethacin, aspirin or ibuprofen) and antioxidants (α-tocopherol acetate and L-ascorbic acid). These combinations resulted in an improvement in the lifespan (77), with synergy demonstrated in animals treated with aspirin and SRT. Injection of neural stem cells into the CNS also synergizes with SRT in Sandhoff disease (78). In this case, the neural stem cells act as a persistent source of enzyme, cross-correcting the surrounding cells (Table 1).

In the mouse model of Niemann-Pick A disease, CNS-directed AAV2-mediated GT combined with an i.v. injection of AAV2/8 expressing human acid sphingomyelinase (ASM) resulted in a synergistic improvement (79). Through this bimodal approach, the group treated the CNS and systemic disease separately, but simultaneously. At 54 weeks of age, survival in the combination treatment group was 100%, while none of the animals that received either CNS-directed AAV2 or systemic AAV2/8 alone survived. Interestingly, in the combination group, no antibodies against human ASM were detected. The AAV2/8 vector utilized a liver-specific promoter which, combined with the strong tropism of AAV2/8 for the liver, reduced the immune response to the enzyme (80). Through this approach, the combination of widespread systemic and CNS expression of ASM, as well as the reduced immune response likely explains the synergy observed in this study.

In the profoundly demyelinating and neurodegenerative disease, globoid-cell leukodystrophy (GLD), several combination therapies have been attempted in the murine model (the twitcher mouse). When BMT is combined with SRT (L-cycloserine), there was an improvement in the mean lifespan to 112 days from ∼50–56 days using either therapy alone (81). Similar results were obtained when CNS-directed AAV2/5-mediated GT was combined with neonatal BMT. There was a dramatic synergy compared to either therapy alone (56), increasing the median lifespan from 45–55 days to 105 days. In a follow-up study, the median lifespan was further increased to ∼130 days when intrathecal administration of AAV2/5 was added to the treatment regimen (Reddy *et al*., manuscript under review). In addition, the dramatic improvement was shown to arise from the immunomodulatory effects of BMT synergizing with the enzyme supplied by GT (Reddy *et al*., manuscript under review). These are striking findings, considering that neonatal BMT alone provides no enzyme to the brain, and minimal clinical benefit (56).

In the murine model of infantile neuronal ceroid lipofuscinosis (INCL; palitoyl protein thioesterase-1, PPT1−/−, deficiency), dramatic synergy is observed when CNS-directed AAV2/5 GT is combined with BMT. Untreated PPT−/− mice have a median lifespan of ∼8 months. CNS-directed AAV2/5-mediated GT alone is modestly effective in this mouse model, extending the median lifespan to ∼14 months. In contrast, BMT alone provides no clinical benefit. Remarkably, the median lifespan in animals receiving both BMT and CNS-directed AAV2/5 GT is ∼17 months (Roberts *et al*., in preparation). In this same model, CNS-directed AAV2/5 in combination with a pharmacologic PPT1 mimetic (cystagon) provides no increase in the lifespan; however, combination-treated animals show improvement in motor function (Macauley *et al*., in preparation).

Although combination therapies are successful in several instances, they may sometimes show no improvement or actually worsen the disease. In the murine model of MPS-IIIB, the combination of CNS-directed AAV2/5 and BMT improved hearing and lysosomal inclusions, but the effects on motor function and lifespan were antagonistic when compared with either therapy alone (82). In this case, the therapies may not provide enough correction to outweigh the deleterious effects of harsh conditioning regimens required for BMT. This study highlights the complex effects of BMT and other therapies on the disease. Similarly, in the murine model of GLD, when *N*-acetyl cysteine (antioxidant) is combined with BMT, no positive effects on lifespan or behavioral performance were observed (Hawkins-Salsbury *et al*., manuscript in preparation). This is surprising considering the dramatic increase in oxidative stress markers in GLD (83–85).

It is not yet possible to predict which combinations will be successful. Undoubtedly, some pathogenic mechanisms are sufficiently down-stream of the primary insult that they will not be good therapeutic targets. A more complete understanding of the mechanisms of the disease in terms of the various pathways that are altered is needed. Also, a better understanding of the mechanism of action of various therapies, such as BMT, SRT or other small molecule drugs, could be helpful in designing more rational combination therapies. It is clear,

however, that in certain instances therapies that show little or no efficacy when used alone, may add to or even synergize with other approaches. Therefore, treatments that have so far provided minimal efficacy *in vivo* (stop-codon read through, molecular chaperones and other small molecule drugs) may need to be revisited in the context of combination therapy. Given the complex nature of LSDs and the limitations of single therapies, it may be necessary to shift focus towards the development of combination treatments in order to more effectively treat these disorders. Ultimately, both single and combination therapies will be most effective if they are initiated before patients become symptomatic, highlighting the need for widespread newborn screening.

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