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ORIGINAL ARTICLE

Improved management of lysosomal glucosylceramide levels in a mouse model of type 1 Gaucher disease using enzyme and substrate reduction therapy

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Abstract Gaucher disease is caused by a deficiency of the lysosomal enzyme glucocerebrosidase (acid β-glucosidase), with consequent cellular accumulation of glucosylceramide (GL-1). The disease is managed by intravenous administrations of recombinant glucocerebrosidase (imiglucerase), although symptomatic patients with mild to moderate type 1 Gaucher disease for whom enzyme replacement therapy (ERT) is not an option may also be treated by substrate reduction therapy (SRT) with miglustat. To determine whether the sequential use of both ERT and SRT may provide additional benefits, we compared the relative pharmacodynamic efficacies of separate and sequential therapies in a murine model of Gaucher disease (D409V/null). As expected, ERT with recombinant glucocerebrosidase was effective in reducing the burden of GL-1 storage in the liver, spleen, and lung of 3-month-old Gaucher mice. SRT using a novel inhibitor of glucosylceramide synthase (Genz-112638) was also effective, albeit to a lesser degree than ERT. Animals administered recombinant glucocerebrosidase and then Genz-112638 showed the lowest levels of GL-1 in all the visceral organs and a reduced number of Gaucher cells in the liver. This was likely because the additional deployment of SRT following enzyme therapy slowed the rate of reaccumulation of GL-1 in the affected organs. Hence, in patients whose disease has been stabilized by intravenously administered recombinant glucocerebrosidase, orally administered SRT with Genz-112638 could potentially be used as a convenient maintenance therapy. In patients naïve to treatment, ERT followed by SRT could potentially accelerate clearance of the offending substrate.

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Competing interests John Marshall, Kerry Anne McEachern, Wei-Lien Chuang, Elizabeth Hutto, Craig Siegel, Ronald Scheule, Diane Copeland and Seng Cheng are all employees and stockholders of Genzyme Corporation. James Shayman has received scientific funding from Genzyme Corporation in the past. Greg Grabowski is a consultant to Genzyme Corporation and has received scientific funding from Genzyme Corporation in the past.

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Abbreviations

CNS	Central nervous system
ERT	Enzyme replacement therapy
ESI/MS	Electrospray ionization mass spectrometry
GD	Gaucher disease
GL-1	Glucosylceramide
H&E	Hematoxylin and eosin
HPLC	High-performance liquid chromatography
NB-DNJ	*N*-butyl deoxynojirimycin
SRT	Substrate reduction therapy

Introduction

Gaucher disease (GD), the most common glycosphingolipid storage disorder, is caused by a deficiency of glucocerebrosidase activity (Brady et al. 1966; Beutler and Grabowski 2001). This results in abnormal accumulation of glucosylcer-

amide (GL-1) in lysosomes of cells from the monocytic/macrophage system and consequent derangement of normal cellular function. Patients can present with a variety of clinical manifestations, including hepatosplenomegaly, thrombocytopenia, anemia, bone disease, pulmonary hypertension and, in more severe cases, central nervous system (CNS) disease. Traditionally, GD patients who have no CNS involvement are classified as having type 1 GD, whereas those exhibiting CNS manifestations are categorized as having neuronopathic GD (type 2 or 3, depending on rate of progression).

Patients with GD are managed principally by enzyme replacement therapy (ERT) with recombinant glucocerebrosidase, (imiglucerase, Cerezyme®) (Weinreb et al. 2002; Grabowski et al. 2009). Imiglucerase improves the visceral, hematological, and skeletal manifestations of patients with type 1 GD but not the CNS manifestations associated with the neuronopathic forms due to the limited ability of the enzyme to traverse the blood–brain barrier. An alternative treatment for adult patients with mild to moderate forms of type 1 GD for whom enzyme replacement therapy is not a therapeutic option is substrate reduction therapy (SRT) using *N*-butyldeoxynojirimycin (NB-DNJ) (miglustat, Zavesca®), which inhibits GL-1 synthase, the enzyme that catalyzes the synthesis of GL-1 (Platt et al. 1997; Cox et al. 2003). However, the responses to therapy with miglustat are reportedly slower and less robust than with imiglucerase (Elstein et al. 2004), and treatment can be associated with adverse side effects (Pastores et al. 2005; Hollak et al. 2009).

In addition to these treatments, a number of other treatment approaches are under consideration for development in the management of GD. An example is the pharmacological chaperoning approach that seeks to promote the folding and trafficking of mutant enzymes to the lysosomal compartment (Steet et al. 2006; Lieberman et al. 2007; Yu et al. 2007). Small-molecule drugs such as isofagomine that may be acting as noncovalent active site inhibitors purportedly harbor these characteristics and can facilitate increased enzyme levels in lysosomes. Potentially complementing this strategy may be the deployment of so-called proteostasis regulators to enhance the concentration of folded mutant enzymes in the endoplasmic reticulum (Mu et al. 2008). Emerging technologies such as gene augmentation therapy (McEachern et al. 2006) and transplantation of gene-modified hematopoietic stem cells (Enquist et al. 2006) represent yet other and perhaps more transformative approaches to addressing this disease. However, these technologies are still at an early and formative stage of development. Finally, there are also efforts aimed at generating biosimilar versions of the approved recombinant enzyme using different expression systems (Elbein et al. 1990; Shaaltiel et al. 2007). Whereas these may offer some advantages in manufacturing, it is unlikely that they will provide any improvements in efficacy (van Patten et al. 2007).

The continuing efforts to identify and develop novel therapeutic approaches for GD are borne of the desire to improve upon the current clinical practice. Included among these is a desire to treat CNS disease, as well as improve therapeutic response in bone and lung. (Futerman et al. 2004; Wraith 2006; Mehta 2008). ERT also engenders production of antibodies to the administered enzyme in a small number of patients (~15%), which can increase the risk of hypersensitivity reactions, although such reactions are rare in GD patients (Starzyk et al. 2007). Furthermore, as most GD patients are treated by intravenously administered enzyme infusions biweekly, therapies that can reduce dependence on enzyme administrations could lessen the burden of treatment as well as the incidence of infusion-associated reactions.

In an attempt to address some of these issues, we examined the relative pharmacodynamic efficacies of following ERT (using recombinant glucocerebrosidase) with a novel drug for SRT ((Genz-112638), in a GD mouse model. The potential of this sequential treatment approach to provide improvement over that attained by the respective monotherapies was highlighted in Sandhoff disease mice, a model for another lysosomal storage disorder (Jeyakumar et al. 2005). However, a recent clinical study in type 1 GD patients using SRT (miglustat) and ERT (imiglucerase) showed that the treatments were not additive, possibly because of partial inhibition of glucocerebrosidase by miglustat. It did suggest that SRT may offer an approach to facilitate maintenance therapy in patients who had been stabilized by imiglucerase (Elstein et al. 2007). The deployment of some small-molecule drugs, such as NB-DNJ, with potential to cross the blood–brain barrier, may also address CNS pathology. Finally, others and we have shown that inhibitors of GL-1 synthase such as Genz-112638 (Zhao et al. 2007) and MZ21 (Aerts et al. 2007) may have the added benefit of treating insulin resistance shown to be associated with some GD patients (Langeveld et al. 2008). Previously, we reported that Genz-112638 is effective in addressing visceral disease in a GD mouse model (D409V/null) (Xu et al. 2003; McEachern et al. 2007). Genz-112638 demonstrates a higher degree of specificity for GL-1 synthase than does NB-DNJ, thereby offering the potential for improved efficacy and fewer side effects. Here, we extended the studies in this murine model to include the use of this effector of SRT following infusions of recombinant glucocerebrosidase.

Methods

Animal studies

Procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee

(IACUC) at Genzyme Corporation following the guidelines issued by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The Gaucher mouse (D409V/null) is a model of type 1 GD exhibiting accumulation of GL-1 in liver, spleen, and lungs but lacking bone or brain pathology (Xu et al. 2003). Animals of both sexes were placed on study at 3 months of age, as previous experiments had indicated that there was no difference in response between males and females to recombinant glucocerebrosidase or Genz-112638. The investigational drug, Genz-112638, is an analog of EtDo-P4 (ethylenedioxy-1-phenylpalmitoylamino-3-pyrrolidino-1-propanol) described initially by Shayman and colleagues (Inokuchi and Radin 1987; Lee et al. 1999). The study had six groups of mice, with group A being sacrificed after 2 weeks to provide baseline levels of tissue GL-1. Groups B, C, and D all received intravenously administered infusions of 10 mg/kg recombinant human glucocerebrosidase synthesized in Chinese Hamster Ovary (CHO) cells (Genzyme Corp., Cambridge, MA, USA) (10 mg/kg) via a tail-vein (100 µl) every 2 days for a total of eight injections. Group B was sacrificed at the end of this regimen (at the same time as group A) to provide enzyme-reduced levels of tissue GL-1 immediately following treatment. Group C received no further treatment and was sacrificed 12 weeks after the start of the study to provide a measure of GL-1 reaccumulation. Groups D and E were both fed Genz-112638 (Genzyme) as a component of the pellet-food diet, with daily dosing starting the day after the last enzyme dose (given to group D). The drug was formulated at 0.075% (w/w) in standard 5053 mouse chow (TestDiet, Richmond, IN, USA) and provided ad libitum. This formulation provided 150 mg/kg of Genz-112638 per day in a 25-g mouse eating 5 g of food per day. The dose of Genz-112638 was selected based on earlier studies (McEachern et al. 2007). Group F received no treatment and was sacrificed along with groups C, D, and E, 12 weeks after the start of the study. Food consumption and mouse weights were monitored three times per week to determine drug intake and the potential impact of the drug on overall health. Animals were killed by carbon dioxide inhalation and their tissues harvested immediately. Half of each tissue was snap frozen on dry ice and stored at –80°C until ready for further processing. The other half was processed for histological analysis.

Quantitation of tissue glucosylceramide levels

GL-1 levels were quantified by mass spectrometry as described previously (McEachern et al. 2006); Doering et al. 1999). A known mass of tissue was homogenized in 2:1 (v/v) chloroform:methanol and incubated at 37°C for 15 min. Samples were centrifuged, and the supernatants were extracted with 0.2 volumes of water overnight at 4°C. The samples were centrifuged, the aqueous phase was discarded, and the organic phase was dried down to a film under nitrogen. For electrospray ionization mass spectrometry (ESI/MS) analysis, tissue samples were reconstituted to the equivalent of 50 ng original tissue weight in 1 ml chloroform:methanol (2:1, v/v) and vortexed for 5 min. Aliquots (40 µl) of each sample were delivered to Waters total recovery vials, and 50 µl of a 10 µg/ml d3-C16-GL-1 internal standard (Matreya, Inc., Pleasant Gap, PA, USA) was added. Samples were dried under nitrogen and reconstituted with 200 µl of 1:4 (v/v) dimethylsulfoxide (DMSO):methanol. ESI/MS analysis of GL-1s of different carbon chain lengths was performed on a Waters alliance high-performance liquid chromatography (HPLC) (Separation Module 2695) coupled to a Micromass Quattro Micro system equipped with an electrospray ion source. Lipid extract samples (20 µl) were injected onto a C8 column (4 ml×3 mm i.d; Phenomenex, Torrance, CA, USA) at 45°C and eluted with a gradient of 50–100% acetonitrile (2 mM ammonium acetate, 0.1% formic acid) at 0.5 ml/min. The first 0.5 min was held at 50% organic and then quickly switched to 100% for the final 3.5 min. The source temperature was held constant at 150°C, and nitrogen was used as the desolvation gas at a flow rate of 670 L/h. The capillary voltage was maintained at 3.80 kV with a cone voltage of 23, and the dwell time for each ion species was 100 ms. Spectra were acquired by the multiple reaction monitoring (MRM) mode to monitor eight dominant isoforms (C16:0, C18:0, C20:0, C22:1, C22:0, C22:1-OH, C24:1, and C24:0). Quantitation of GL-1 was based on the sum of these eight isoforms relative to the internal standard, with a calibration curve ranging from 0.1 to 10 µg/ml.

Histology

For histological analysis, tissues were fixed in zinc formalin (Electron Microscopy Sciences, Hatfield, PA, USA) at room temperature for 24 h, then stored in phosphate-buffered saline (PBS) at 4°C until ready for further processing. All samples were dehydrated in ethanol, cleared in xylenes, infiltrated, and embedded in Surgipath R paraffin (Surgipath, Richmond, IL, USA). Five-micron sections were cut using a rotary microtome and dried in a 60°C oven prior to staining. Sections were deparaffinized in Hemo-De (Scientific Safety Solvents, Keller, TX, USA) and rehydrated in descending concentrations of ethanol followed by a wash with PBS. The sections were stained with hematoxylin and eosin (H&E) and labeled using a rat anti-mouse CD68 monoclonal antibody (Serotec, Raleigh, NC, USA) to identify macrophages. After washing for 5 min in PBS, the slides were dehydrated in ethanol and cleared in Hemo-De prior to mounting with SHUR/Mount™ coverglass mounting medium (TBS, Durham,

NC, USA). The percent area of CD68 immunopositivity in the liver was quantified using MetaMorph (MDS Analytical Technologies, Toronto, Canada) analysis of ten separate 400× images per tissue section. A board certified veterinary pathologist blinded to group designation examined all sections.

Results

Dosing regimen of glucocerebrosidase for debulking accumulated GL-1 in the liver, spleen, and lung of 3-month-old Gaucher mice

To investigate the relative merits of sequential therapy and monotherapy with enzyme (recombinant glucocerebrosidase) or substrate reduction (Genz-112638), we first determined the enzyme regimen that maximally depleted GL-1 levels in the visceral organs of Gaucher mice. Three-month-old Gaucher mice (D409V/null) were intravenously administered two, four, or eight doses of 10 mg/kg recombinant human glucocerebrosidase. The mice treated with two or four doses of the enzyme received drug infusions every 3 days; those treated with eight doses received the enzyme every 2 days. The use of a shorter time interval between infusions in animals that received eight treatments was designed to minimize the potential impact of any immune response to the administered human enzyme on efficacy. The animals were killed 7 days following the last enzyme infusion, and the amount of GL-1 remaining in their livers, spleens, and lungs was measured.

Treatment with two doses of glucocerebrosidase reduced the levels of GL-1 in the liver by 50% (Fig. 1). Increasing the number of enzyme infusions to four or eight, as expected, reduced liver GL-1 levels to a greater extent (by approximately 75%). The less than complete lowering of GL-1 levels, even with eight doses, is consistent with the experience in Gaucher patients where hepatosplenomegaly is reduced only after an extended period of treatment (Grabowski et al. 1995). The substrate levels in the spleens of Gaucher mice were more refractory to enzyme treatment. Administration of two doses of glucocerebrosidase did not significantly alter GL-1 levels from those noted in untreated controls (Fig. 1). Increasing the number of enzyme infusions to four or eight reduced splenic GL-1 levels by about 50%. In the lung, a reduction to approximately 60% of untreated control was observed after eight doses. The slightly lower extent of substrate reduction in the lung was probably due to poorer accessibility of the infused enzyme to the lipid-laden alveolar macrophages. The observation of greater GL-1 clearance in the liver when compared with the spleen and lung likely reflects the biodistribution of the enzyme following systemic infusion (van Patten et al. 2007). Based on these results, the treatment regimen consisting of eight consecutive doses of 10 mg/kg glucocerebrosidase administered at 2-day intervals was used for subsequent studies.

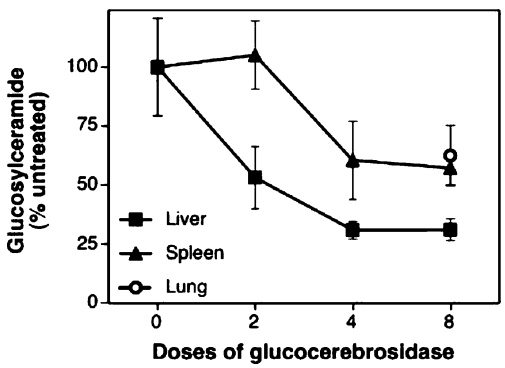

Fig. 1 Effect of administering exogenous glucocerebrosidase on glucosylceramide (GL-1) levels in liver, spleen and lung. Three-month-old Gaucher mice were administered 10 mg/kg recombinant glucocerebrosidase intravenously two, four, or eight times. The two- and four-dose cohorts received the enzyme 3 days apart, and the group treated with eight doses was injected 2 days apart. GL-1 levels in the liver (*squares*), spleen (*triangles*), and lung (*circle*) were measured 7 days after the last injection. Data are expressed as means ± standard error of the mean (SEM) (n=5)

Relative abilities of enzyme and substrate reduction therapy to lower GL-1 levels in the liver of Gaucher mice

Cohorts of 3-month-old Gaucher mice were treated with either recombinant glucocerebrosidase (Fig. 2, columns B & C) or Genz-112638 (inhibitor of GL-1 synthase) separately (Fig. 2, column E) or sequentially (Fig. 2, column D). Mice in groups B, C, and D were given eight doses of enzyme as described above (over a period of 2 weeks) to clear accumulated GL-1. Different groups were then fed either regular chow or chow containing Genz-112638 (150 mg/kg per day) for an additional 10 weeks, with group F receiving no treatment and serving as the control. Irrespective of the chow formulation, the mice ate comparable amounts of food, and there were no discernible differences in weight gain (data not shown). Administration of Genz-112638 at the dose indicated was well tolerated, with no notable effects on the mice. Approximately 80% of the stored GL-1 levels were cleared from the liver following 2 weeks of enzyme therapy alone (Fig. 2, column B). When these animals were allowed to progress without further treatment for 10 weeks, their liver GL-1 levels increased, indicating that reaccumulation of the substrate had occurred during the intervening period (Fig. 2, column C). These levels were not significantly different from those of untreated controls (Fig. 2, column F). However, if the mice were treated first with enzyme for eight doses and then with Genz-112638 in their food over a 10-week

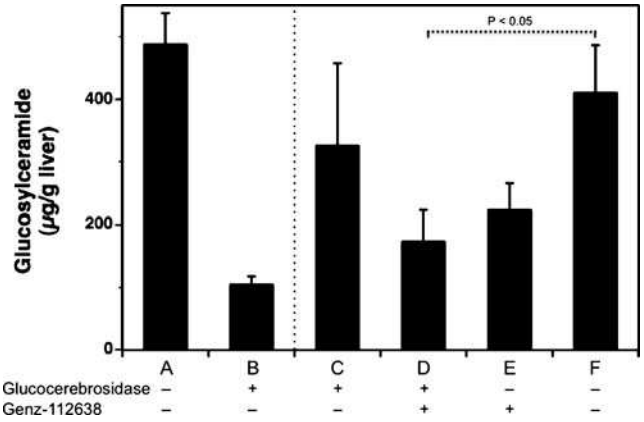

Fig. 2 Efficacy of enzyme and substrate reduction therapies at lowering glucosylceramide (GL-1) levels in the liver of Gaucher mice. Liver GL-1 levels were measured in untreated 3-month-old Gaucher mice (**A**) and following 2 weeks of treatment with recombinant glucocerebrosidase (**B**). Mice treated with recombinant glucocerebrosidase were analyzed 10 weeks later without further treatment (**C**) or after therapy with glucosylceramide synthase inhibitor (Genz-112638) (**D**). GL-1 levels in the liver of mice administered Genz-112638 alone for the entire study period (**E**) and in untreated, age-matched controls (**F**) are also shown. Data are expressed as means ± standard error of the mean (SEM) (n=5). Statistical significance was determined using the unpaired t test

period, their liver GL-1 levels were significantly lower than the untreated controls (Fig. 2, columns D & F). This result suggests that the additional treatment with Genz-112638 slowed substrate reaccumulation. Interestingly, Gaucher mice treated with Genz-112638 alone during the entire study period (12 weeks) also showed lower GL-1 levels (Fig. 2, column E) when compared with untreated, age-matched controls (Fig. 2, column F), though the difference was not statistically significant. The ability of SRT alone to reduce GL-1 levels in this animal model is consistent with our previous report (McEachern et al. 2007) and likely reflects the fact that Gaucher mice (D409V/null) retain residual enzymatic activity (Xu et al. 2003).

Relative abilities of enzyme and substrate reduction therapy to lower GL-1 levels in the spleen of Gaucher mice

Treating 3-month-old Gaucher mice with recombinant glucocerebrosidase alone for 2 weeks reduced splenic GL-1 levels by approximately 60% (Fig. 3, column B). When these animals were allowed to age for an additional 10 weeks without further intervention, the substrate levels returned to those observed at the start of the study (Fig. 3, column C) and were not significantly different from the untreated control (Fig. 3, column F). This suggests that the rate of reaccumulation of GL-1 in the spleen was higher than in the liver. This supposition was also supported by the observation of higher basal levels of the substrate in the spleen (~1,500 μg/g tissue; Fig. 3, column A) than in the liver (~500 μg/g tissue; Fig. 2, column A). Animals treated with enzyme for eight doses and then with Genz-112638 for the next 10 weeks showed the greatest reduction in splenic GL-1 levels (Fig. 3, column D), and these were significantly lower than those in the untreated control spleens (Fig. 3, column F). This indicated that the deployment of Genz-112638 not only delayed the reaccumulation of substrate but also acted to further reduce the burden of storage in this organ. It would appear that at least in this instance, the net effect of the residual endogenous enzyme and substrate reduction led to a further decline in overall substrate levels. The observation of lower splenic GL-1 levels in mice treated with Genz-112638 alone for 12 weeks (Fig. 3, column E) than in untreated controls (Fig. 3, column F) is consistent with this notion, though the difference was not significant. Hence, in mild type 1 GD patients with residual enzyme activity, treatment with imiglucerase followed by Genz-112638 could potentially accelerate the rate and perhaps even increase the extent of clearance of the offending substrate.

Relative abilities of enzyme and substrate reduction therapy to lower GL-1 levels in the lung of Gaucher mice

As noted earlier, pulmonary GL-1 levels were least effectively cleared by intravenous administration of recombinant glucocerebrosidase. Treatment of 3-month-old Gaucher mice with enzyme for 2 weeks resulted in only a 30% reduction in substrate levels in the lung (Fig. 4,

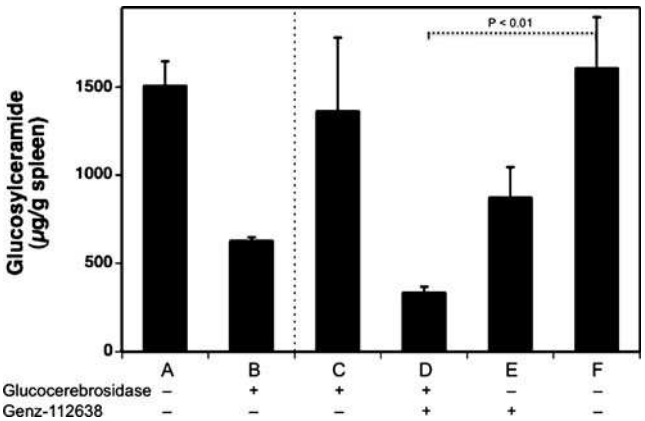

Fig. 3 Efficacy of enzyme and substrate reduction therapies at lowering glucosylceramide (GL-1) levels in the spleen of Gaucher mice. Spleen GL-1 levels were measured in untreated 3-month-old Gaucher mice (**A**) and following 2 weeks of treatment with recombinant glucocerebrosidase (**B**). Mice treated with recombinant glucocerebrosidase were analyzed 10 weeks later without further treatment (**C**) or after therapy with glucosylceramide synthase inhibitor (Genz-112638) (**D**). GL-1 levels in the spleen of mice administered Genz-112638 alone for the entire period of study (**E**) and in untreated, age-matched controls (**F**) are also shown. Data are expressed as means ± standard error of the mean (SEM) (n=5). Statistical significance was determined using the unpaired t test

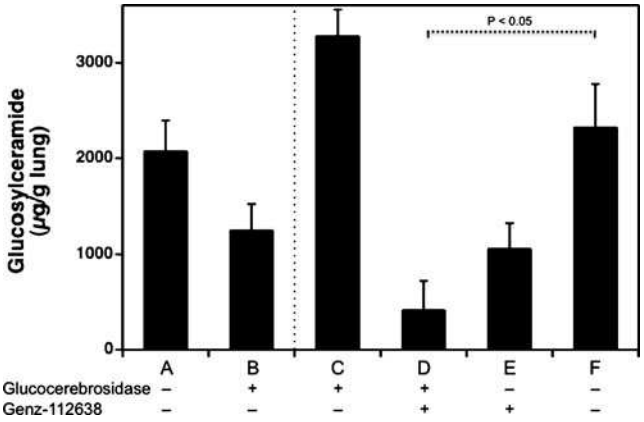

Fig. 4 Efficacy of enzyme and substrate reduction therapies at lowering glucosylceramide levels in the lung of Gaucher mice. Lung glucosylceramide (GL-1) levels were measured in untreated 3-month-old Gaucher mice (**A**) and following 2 weeks of treatment with recombinant glucocerebrosidase (**B**). Mice treated with recombinant glucocerebrosidase were analyzed 10 weeks later without further treatment (**C**) or after therapy with glucosylceramide synthase inhibitor (Genz-112638) (**D**). GL-1 levels in the lung of mice administered Genz-112638 alone for the entire study period (**E**) and in untreated, age-matched controls (**F**) are also shown. Data are expressed as means ± standard error of the mean (SEM) (n=5). Statistical significance was determined using the unpaired t test

column B). The cohort of animals fed normal chow for the ensuing 10 weeks showed, as expected, reaccumulation of GL-1 to untreated levels (Fig. 4, column C & F). In contrast, animals fed chow containing Genz-112638 over the same intervening period showed a reduction in substrate levels to below those administered enzyme alone (Fig. 4, column D) and were significantly lower than those in the untreated controls (Fig. 4, column F). Again, this suggests that in the lung, as in the spleen, the net effect of Genz-112638 (in the presence of residual endogenous enzyme activity) not only retarded the reaccumulation of GL-1 but also acted to further reduce it to below starting levels. As with the other visceral organs, treatment with Genz-112638 alone was effective in lowering pulmonary GL-1 levels (Fig. 4, column E) when compared with untreated controls (Fig. 4, column F).

Histopathological analysis of the liver of Gaucher mice after enzyme and substrate reduction treatment

To visualize the effects of the different therapeutic regimens in the liver, tissue sections were stained for CD68, a macrophage marker. Analysis of liver sections from untreated 3-month-old Gaucher mice showed the presence of large numbers of lipid-engorged, CD68-positive Gaucher cells (Fig. 5, panel a) that remained largely unchanged when analyzed 12 weeks later (Fig. 5, panel f). Consistent with the biochemical data above, livers of animals administered recombinant glucocerebrosidase over a period of 2 weeks showed substantial clearance of the lipid in these abnormal macrophages (Fig. 5, panel b). If these animals were allowed to age an additional 10 weeks without further treatment, there was evidence of reaccumulation of GL-1 as indicated by the reemergence of Gaucher cells (Fig. 5, panel c). However, this increase in Gaucher cells was negated if the mice were given substrate reduction therapy with Genz-112638 over the same intervening period (Fig. 5, panel d). As noted earlier, Gaucher mice that received Genz-112638 alone also showed reduced accumulation of the substrate (Fig. 5, panel e), although not to the same degree as those that received sequential treatment with recombinant glucocerebrosidase followed by Genz-112638. The extent of CD68-positive staining on the various sections was also quantified using MetaMorph software (Fig. 6). The degree of staining in these sections mirrored the amounts of liver GL-1 levels determined biochemically (Fig. 2), further supporting suggestions on the relative merits of the different treatment regimens.

Discussion

Extensive clinical experience with ERT using recombinant glucocerebrosidase (alglucerase or imiglucerase) indicates that this is an effective therapy for patients with type 1 GD. Treatment with imiglucerase results in a reduction or normalization of hepatosplenomegaly, a lower frequency of bone pain and bone crises, and improvement in a variety of hematological and biochemical biomarkers (Weinreb et al. 2002; Grabowski et al. 2009; Charrow et al. 2004; de Fost et al. 2006). Studies to optimize dosing of ERT for type 1 GD and maximize patient convenience continue (Grabowski et al. 2009; de Fost et al. 2006; Weinreb et al. 2008; Zimran et al. 2009; Kishnani et al. 2009). Whereas a consensus has not yet been reached, it is apparent that higher doses and more frequent infusions generally result in more rapid reversal of several disease parameters (Grabowski et al. 2009).

SRT with miglustat is also available to type 1 GD patients with mild to moderate disease for whom ERT is not a therapeutic option. The SRT approach seeks to retard synthesis of glycosphingolipids to rates at which the residual enzymatic activity is able to catabolize stored and incoming GL-1 (Platt et al. 1997; Inokuchi and Radin 1987). Clinical studies indicate that in addition to offering the convenience of oral therapy, SRT could also reduce the burden of GL-1 storage, with a resultant improvement in disease manifestations, albeit over a longer period of treatment than with ERT (Cox et al. 2003; Elstein et al. 2007; Pastores et al. 2009). Moreover, a study of patients treated by ERT and then switched to SRT with miglustat suggests that the latter might be deployed in the context of

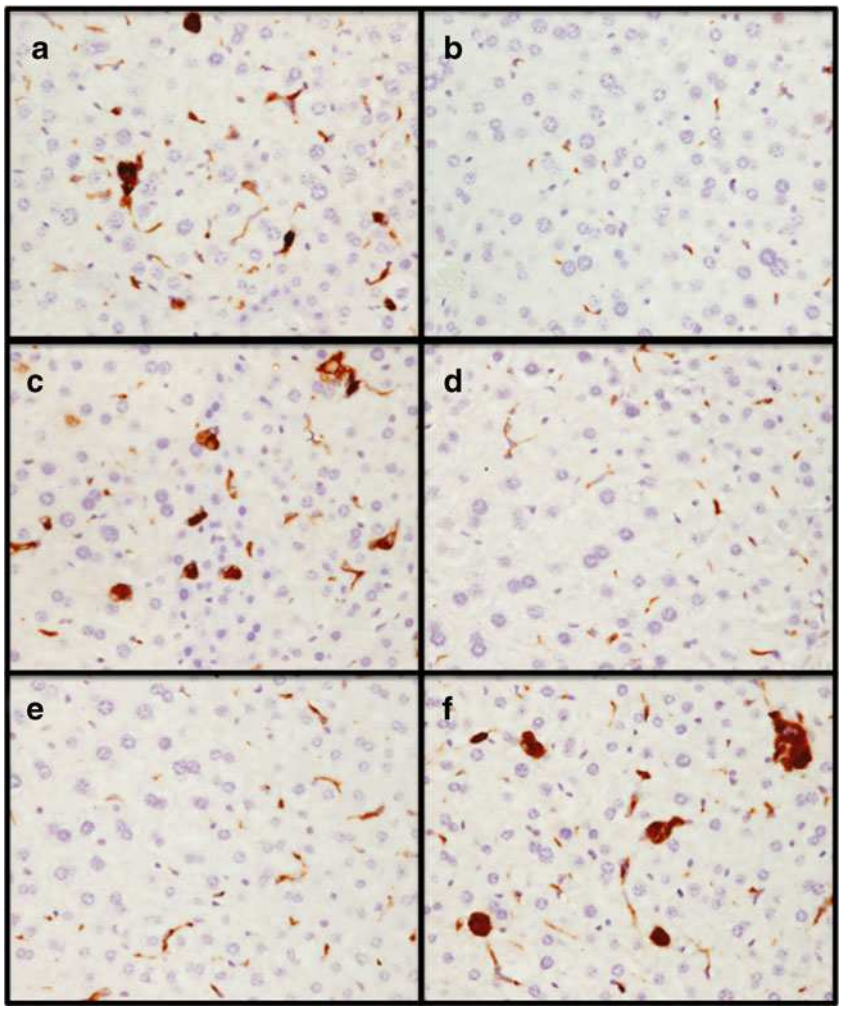

Fig. 5 Immunohistochemical staining of liver of Gaucher mice following treatment with enzyme (ERT) and substrate reduction therapy (SRT). Liver sections were stained with an anti-CD68 antibody to visualize macrophages. Sections shown were from untreated 3-month-old Gaucher mice (**a**), 3-month-old Gaucher mice dosed with glucocerebrosidase and analyzed after 2 weeks of treatment (**b**), or 10 weeks later (**c**). Liver of Gaucher mice administered enzyme followed by glucosylceramide synthase inhibitor (Genz-112638) (**d**), and from those that received Genz-112638 alone (**e**), liver of untreated Gaucher mice at the end of the study (12 weeks later) (**f**)

maintenance therapy for GD patients, particularly those whose disease has been stabilized by ERT (Elstein et al. 2007; Giraldo et al. 2006). However, a combination of both ERT with imiglucerase and SRT with miglustat reportedly did not provide additional benefits (Elstein et al. 2007), possibly due to nonspecific inhibition of glucocerebrosidase by NB-DNJ. The premise for the study presented here was predicated on the notion that because the two therapeutic strategies have dissimilar modes of actions, they might be additive. Evidence of different therapies showing benefit were illustrated in an animal model of Sandhoff disease, another lysosomal storage disorder, whereby a combination of bone marrow transplantation (to provide enzyme) and SRT (NB-DNJ) was more effective than either treatment alone (Jeyakumar et al. 2001). Moreover, as the biodistribution and pharmacodynamics of a small-molecule drug are different from enzyme, the hope was that SRT might address diseased organs that were less amenable to ERT, such as bone, lung, and even the central nervous system (CNS).

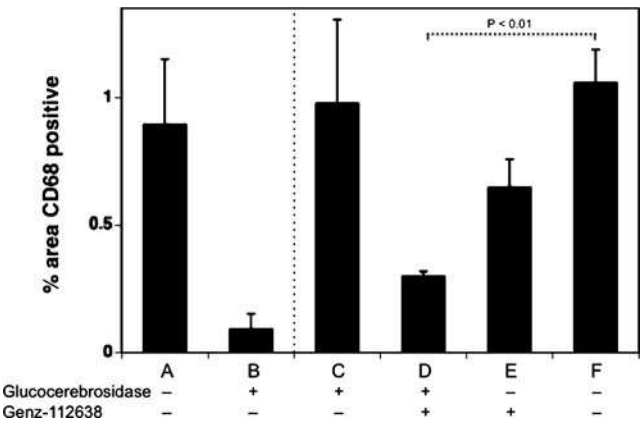

Fig. 6 The extent of CD68-positive staining on liver sections was quantified using MetaMorph software. Levels in untreated 3-month-old Gaucher liver (**A**) or following treatment with glucocerebrosidase (**B**). Mice treated with enzyme and then analyzed 10 weeks later without further therapeutic intervention (**C**) or after therapy with glucosylceramide synthase inhibitor (Genz-112638) (**D**). Extent of staining in the liver of Gaucher mice administered Genz-112638 alone (**E**) and in untreated, age-matched control mice (**F**). The data was collated from an analysis of ten 400× images per section from each mouse. Statistical significance was determined using the unpaired *t* test

Two small-molecule drugs, one approved (NB-DNJ) and the other in phase 3 clinical testing (Genz-112638), have been developed for SRT of type 1 GD. Both inhibit GL-1 synthase, the enzyme that catalyzes the first step in the biosynthesis of glycosphingolipids. Genz-112638, an investigational drug, was developed with the goal of reducing some of the purported off-target effects shown to be associated with NB-DNJ, which could lead to gastrointestinal distress, tremor, and peripheral neuropathy (Cox et al. 2003; Pastores et al. 2009). We reported previously that Genz-112638 exhibits high potency and specificity at inhibiting GL-1 synthase and is effective at reducing lysosomal accumulation of GL-1 in a murine model of GD (McEachern et al. 2007). We extended these studies to ascertain the relative merits of its use after initial enzyme administration in the same murine model. Although in the original murine studies Genz-112638 was administered via oral gavage, bridging studies have generated similar results when the inhibitor was delivered as a component of the chow.

The primary objective of the studies was to determine whether introducing SRT after ERT could improve upon the pharmacodynamic efficacy observed with either therapy alone or maintain the efficacy observed with ERT alone. As expected, treatment with either modality alone was effective in reducing substrate accumulation in all the visceral organs tested. As such, they support the clinical findings noted in type 1 GD patients. Moreover, there was a sustained reduction of GL-1 levels observed in the visceral organs of mice treated with Genz-112638 after prior substrate clearance by ERT. Importantly, mice that received sequential treatment with recombinant glucocerebrosidase and Genz-112638 showed greater substrate clearance than realized with either treatment alone. This would suggest that the sequential use of the two therapeutic modalities was additive. Hence, a sequential treatment approach may be considered in instances where rapid debulking of accumulated GL-1 is desirable, as may occur in adult type 1 GD patients who are naïve to treatment. However, as noted earlier, a study with sequential recombinant glucocerebrosidase (imiglucerase) and miglustat, albeit in a small number of GD patients, showed no additional benefit (Elstein et al. 2007). Hence, the translatability of these observations in mice to humans is yet to be validated. Moreover, as miglustat was associated with some side effects in GD patients, a better understanding of whether this was specifically associated with the drug or if this was a class effect needs to be determined.

In summary, the data presented here demonstrate that in mice, treatment with ERT using recombinant glucocerebrosidase followed by SRT using Genz-112638 may be an effective approach to managing the substrate that accumulates in the visceral organs of nonneuropathic GD patients. These findings support the design of a proposed phase 3 trial in humans that will evaluate Genz-112638 in patients previously treated with imiglucerase. During the execution of these studies, another mouse model of GD that is more reflective of the disease manifestations in patients was generated (Enquist et al. 2006). Additional testing of this treatment paradigm in the more severely affected mouse model is warranted to further ascertain the merits of this strategy. Moreover, as Genz-112638 is an inhibitor of GL-1 synthase that initiates the glycosphingolipid biosynthetic pathway, this treatment paradigm may also be evaluated in other glycosphingolipid storage diseases, such as Fabry disease.

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