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Delivery of lysosomal enzymes for therapeutic use: glucocerebrosidase as an example

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Enzyme therapies for lysosomal storage diseases have developed over the past decade into the standard-of-care for affected patients. Such therapy for Gaucher disease has been the prototype, using natural source or recombinant forms of human acid β-glucosidase (GCase). In Gaucher disease, macrophages are the repository for the pathological lipid and the target for delivery of GCase. The macrophage mannose receptor provides a Trojan horse for intracellular delivery of intravenously administered GCase (man-GCase) with mannosyl-terminated oligosaccharide chains. Passage through several hostile compartments (e.g., plasma) leads to inefficient delivery of man-GCase to macrophage lysosomes. However, regular infusions of man-GCase re-establishes health in affected patients. Similar results are being obtained in several other lysosomal storage diseases. Evolving gene and chaperone approaches provide alternative treatment strategies.

Keywords: enzyme replacement therapy, Gaucher disease, inborn errors of metabolism, lipidosis, sphingolipidosis

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1. Introduction

Glucocerebrosidase, acid β-glucosidase (GCase), is the penultimate enzyme in the degradation of glycosphingolipids containing a β-glucose core attached to ceramide: the core sphingolipid (**Figure 1**). Interest in GCase stems from its defective function in the lysosomal storage disease (LSD), Gaucher disease being the most common [1]. Although panethnic, the trait has high frequency in the Ashkenazi Jewish population, and has been the prototype for the development of enzyme therapies for the LSDs. Indeed, the medical and economic success of enzyme therapy for Gaucher disease provided the foundation for the development, and FDA and European Medicines Evaluation Agency approval, of such therapies for Fabry disease, mucopolysaccharidoses (MPS) I, II and VI, and Pompe disease (glycogenosis type II) [2-5].

A short description of Gaucher disease and its pathology provide a background for understanding the issues of drug delivery for LSDs and, in particular, Gaucher disease. The disease is autosomal recessively inherited and has a frequency in the general population of ~ 1/75,000 – 1/40,000. It has its highest frequency (1/400 – 1/1000) among Ashkenazi Jews [1]. The most prevalent variant of Gaucher disease is termed the non-neuronopathic or type 1 variant, as it does not manifest primary involvement of the CNS [1]. The major organs involved include the liver, spleen, bone and bone marrow and, less frequently, the lungs [6]. Within these tissues, only selected cell types are involved by the massive storage of glucosyl ceramide (**Figure 1**), the substrate for GCase. These cells are derived primarily from the monocyte/macrophage system, and, thus, Gaucher disease type 1 is primarily a disorder of macrophages. The inability to cleave the requisite amount of glucosyl ceramide leads to the excess accumulation of this substrate within macrophages and other cells of the reticuloendothelial system. The major sources of the glucosylceramides are membranes of senescent circulating red

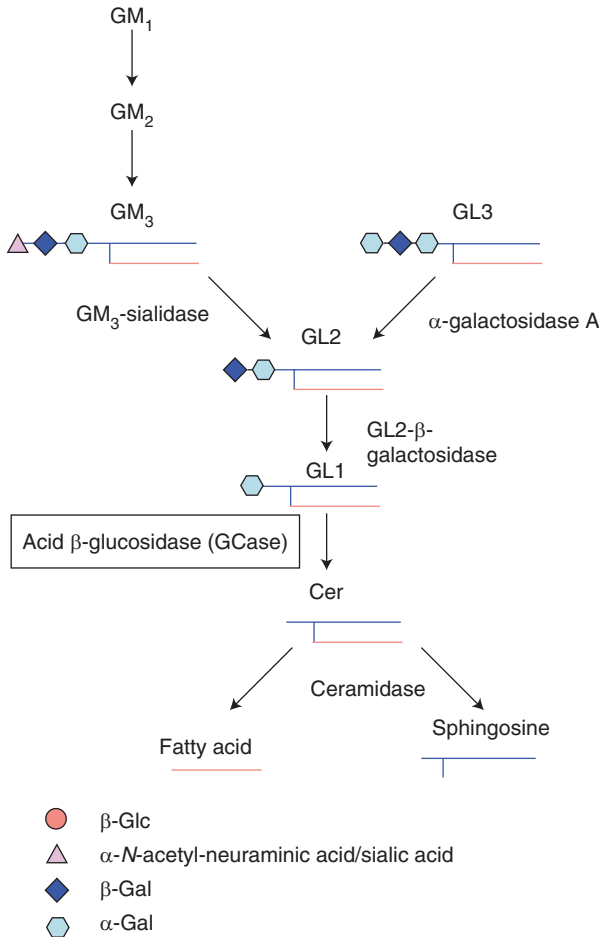

Figure 1. Schematic of the glycosphingolipid degradative pathway in lysosomes. GM_x refers to gangliosides where x = 1, 2 or 3. GLx refers to neutral glycosphingolipids where x = 1, 2 or 3 or glucosylceramide, lactosylceramide or globotriaosylceramide, respectively. Cer is ceramide containing a sphingosyl (blue) and fatty acid acyl (red) chain. The coloured symbols attached to Cer represent various glycons including β-glucose, β-galactose, α-galactose and α-*N*-acetyl-neuraminic acid. GL3 and GL1 accumulate in Fabry and Gaucher diseases, respectively, due to the deficiency of the respective lysosomal hydrolases.

and white blood cells that are phagocytosed by macrophages [7]. Histological examination reveals characteristic engorged macrophages that contain fibular deposits (**Figure 2**) in almost crystalline bilaminar structures [8] (the stored substrate). Through poorly described mechanisms, the accumulation of glucosyl ceramide within the macrophages leads to the activation of cytokine and chemokine pathways that exert detrimental pro- and anti-inflammatory effects within tissues, leading to a variety of tissue involvement including fibrosis, necrosis, haemorrhagic infarction and frank necrosis [9]. The mechanisms by which these events occur are not well described, but involve programmed cell death pathways including apoptosis.

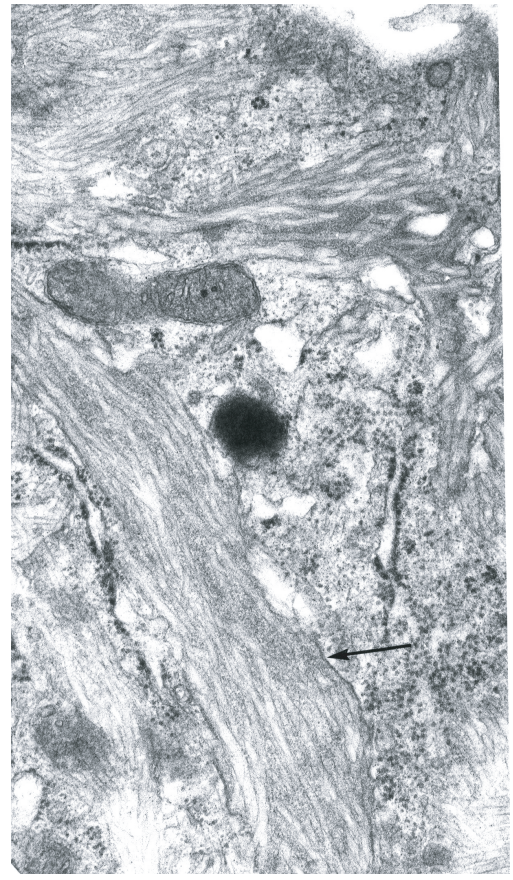

Figure 2. Electron micrograph of the characteristic deposits within Gaucher cells. The tubular appearing structures are composed mostly of glucosyl ceramide and are typical of Gaucher disease.

The fundamental concept of enzyme therapy for Gaucher disease and other LSDs was inherent in the description of the lysosome as a subcellular organelle [10]. De Duve first suggested the need for lysosomal enzymes to be specifically localised to this organelle to avoid digestion of essential cellular components. Thus, a targeting system was necessary for the compartmentalisation within the lysosome of synthesised enzymes from nuclear encoded proteins. In addition, as all surviving Gaucher disease patients have at least some residual enzyme activity, enzyme therapy for the disease must include the concept of delivering sufficient supplementary enzyme to the lysosome to normalise metabolism and flux within the lysosome of the glucosyl ceramide. The amount of enzyme needed to affect treatment is probably tissue specific and not well defined (see below). Small amounts of residual enzyme activity (~ 0.5% of wild type) were required to normalise cellular metabolism in MPS I cells and such low levels were evident in the threshold hypothesis of Sandhoff and co-workers [11-13]. These latter investigators showed that small incremental changes in the amount of enzyme activity could modify the

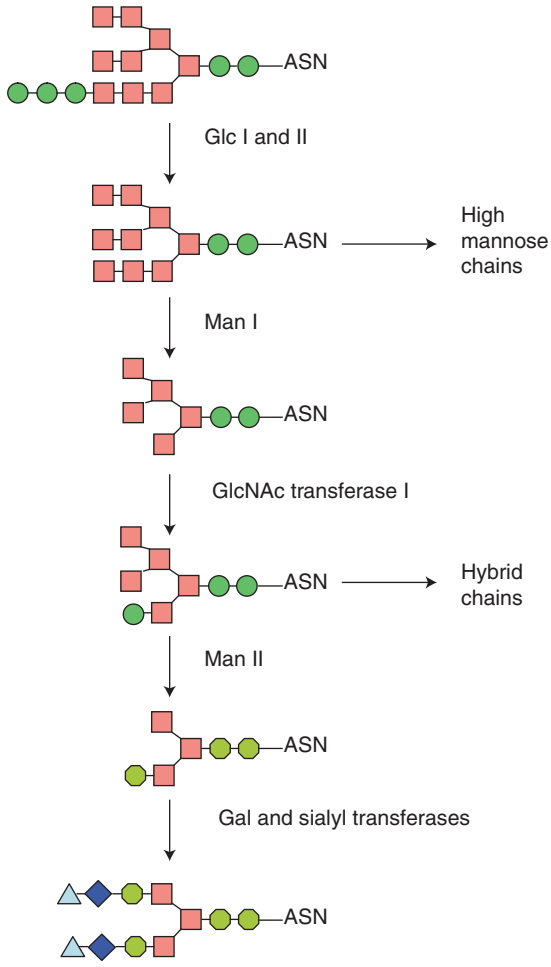

Figure 3. Schematic of the oligosaccharide processing pathway for *N*-linked glycoproteins. Glc I and II, Man I, GlcNAc transferase I, Man II, and Gal and Sialyl transferases are resident enzymes in various cellular compartments for the removal or addition of specific glycon species from the backbone chain. Glc I and II reside in the ER. Man I and II are resident in the *cis*- and mid-Golgi, respectively. GlcNAc transferase I, Gal and α-sialyl transferases (Sialyl) are resident in the mid- and *trans*-Golgi, respectively.

Gal: β-Galactose; Glc: α-Glucosidase; GlcNAc: *N*-acetyl-glucosamine; Man: α-Mannosidase.

clinical phenotype from severe/lethal infantile onset phenotypes to later onset adult variants, and to unaffected heterozygotes for various lysosomal diseases. Therefore, small incremental changes in enzyme amounts within lysosomes could be therapeutic and lead to effective therapy under all but highly stressful cell conditions.

2. Detailed description of β-glucosidase

GCase is expressed from the GBA locus on human chromosome 1p21.1 [14]. The transcriptional/translational control of GCase/GBA expression has not been well studied, but elements in the first intron and exon could modulate differential expression of GCase mRNA in various tissues [15,16]. This may account for the differences in GCase-specific activities in CNS, skin fibroblasts and placenta (high) and lymphocytes and monocytes (low). Xu *et al.* showed the presence of a dsRNA binding protein termed ILF3/TCP80 that binds to the GCase mRNA within the coding region and traps or prevents mRNA attachment to polysomes for translation [17-19]. Control of this mechanism is poorly understood, but the PKC phosphorylation is involved in the pathway [17].

In comparison, much more is known about the properties of the GCase protein, its requirements for activity, and the modulation of its function within cells. GCase is a tightly membrane-associated lysosomal protein that is synthesised in the endoplasmic reticulum. Signal sequence clipping and the nascent protein glycosylation occur co-translationally during penetration through the ER membrane [20-24]. Transit from *cis* to *trans*-Golgi leads to sequential modification of the *N*-linked oligosaccharides attached to this glycoprotein, from high mannose to complex forms (**Figure 3**). The enzyme does not require additional proteolytic processing following signal sequence clipping, and appears to be monomeric in its fully active form. Thus, macromolecular assembly or subunit association is not needed for maturation or targeting to the lysosome. Unlike many of the soluble lysosomal enzymes that use the mannose-6-phosphate system for intracellular targeting to the lysosome, GCase does not require oligosaccharides [23,25]. This implicates an internal targeting peptide sequence that has not been fully characterised [25], but appears to have a unique structure unlike those within other integral lysosomal membrane proteins (Grabowski *et al.*, unpublished observation). In human cultured skin fibroblasts, the transit time for GCase from the endoplasmic reticulum through the Golgi to the lysosome is ~ 2 – 3 h, and its residence time in lysosomes is ~ 34 – 40 h [20,22]. Within macrophages and monocytes, the enzyme appears to have a $t_{1/2}$ value of ~ 60 h [26]. The lifetimes of GCases in other cell types have not been defined with any precision.

The oligosaccharides are attached to GCase through *N*-glycosylation sites [27,28]. Four of the five *N*-glycosylation sites (i.e., N-X-S/T) on the enzyme are occupied, and the site near the carboxy terminal end (N462) is never occupied [28]. The role of these oligosaccharide side chains appears similar to those on other lysosomal proteins that protect the enzyme from proteolytic degradation. For GCase, the occupancy of the first glycosylation site (N19) during protein synthesis is essential for the development of a catalytic active conformer [28]. The absence of glycosylation at N19 during synthesis leads to a catalytically inactive enzyme in fibroblasts and other recombinant cell systems [28]. However, once formed, the fully glycosylated enzyme can be, under appropriate conditions, deglycosylated to retain full activity [29]. The resultant deglycosylated enzyme is significantly sensitive to denaturation by alkaline pH and care must be taken to preserve activity. Glycosylation at the

four *N*-glycosylation sites is important for the development of a stable active form of the enzyme, but is not apparently needed for the maintenance of activity *in vitro* or *in vivo*.

The lack of a role for oligosaccharide side chains on GCase in lysosomal targeting of the newly synthesised enzyme must be distinguished from the Trojan horse receptor – the macrophage mannose receptor (MMR) [30-33] – used for delivery of exogenous enzyme through the plasma membrane to the lysosome for enzyme therapy (see Section 4).

3. Control of β-glucosidase activity

GCase is a monomeric, diprotic enzyme with a bell-shaped pH optimum curve, and *in vitro* maximal activity occurs at pH ~ 4.4 – 5.5, depending on assay conditions [34,35]. The *in vitro* activity requires the presence of detergents or negatively charged phospholipids, in particular phosphatidylserine, for optimal activity (see [34,35] for review). GCase within cells is thought to require these phospholipids for activity, but the identity of the natural phospholipid(s) is unknown. Significant literature is available on the characterisation of the GCase in its purified form (see [1,35] for review). The majority of purified enzymes have been delipidated using organic solvents to strip all endogenous adherent lipids. The enzyme does not contain covalently attached lipids and can be completely delipidated with such solvents. In a delipidated state, the purified enzyme has essentially no activity toward any substrate, and reactivation requires negatively charged phospholipids or detergents. Additional enhancements of activity are obtained by adding a small 80 amino acid peptide, termed saposin C, to the phospholipid-activated GCase [36]. Saposin C is a natural activator of the enzyme and is encoded by a single gene, termed prosaposin, that also encodes three other activators (~ 80 amino acids in length) for different glycosphingolipid hydrolytic enzymes. These other saposins are termed A, B and D [37,38]. Thus, a single locus provides a transcript and a peptide that, by proteolytic processing, provides control over the entire glucosphingolipid degradative pathway through the modulation of various hydrolytic enzymes [39,40]. The saposin C activation mechanism of GCase is not fully detailed, but a significant conformational change in the enzyme has been demonstrated by circular dichroism spectroscopy, thereby conforming the enzyme into an optimal conformer for substrate hydrolysis after interaction with a negatively charged phospholipid [36]. The essential need for saposin C was shown in patients who lack this protein and develop a Gaucher-like disease [41].

In the past 4 years, three crystal structures of wild-type human GCase have become available. Those from the Sussman group and by Liou *et al.* [42-44] have been based on partially deglycosylated GCase. Similar structures were obtained and contain three domains that include domain 1 (amino acids 1 – 25 and 382 – 413), an immunoglobulin-like domain (domain 2, amino acids 26 – 76 and 431 – 497) and the active site domain (domain 3, amino acids 77 – 381 and 414 – 430) (**Figure 4**). By functional assessment, active site labelling and crystallisation with an active-site-directed inhibitor, conduritol B epoxide, the acid/base and catalytic nucleophile in catalysis have been identified as E235 and E340, respectively [43-45]. Additional crystal structures have shown significant loop movement around the active site during binding of a potent (K_i ~ 20 nM) reversible inhibitor, isofagomine (Liebermann, Ringe and Petsko, unpublished observation). Molecular docking with this later structure conforms well to the kinetically predicted conformation of the active site that included binding sites for the glucose head group, and individual subsites for binding of the sphingosyl and fatty acid acyl chain of glucosyl ceramide [46,47].

The disulfide bonds between cysteines 4 and 18, and 16 and 23 are essential for activity [44]. The other three cysteines at C126, 248 and 342 are free, but require a specific oxidation state for activity. C342 is located near the catalytic nucleophile, and is preserved throughout phylogeny from man to worm [44]. Site-directed mutagenesis of C128 or C248 with a C to S substitution led to minor changes in enzymatic activity. The importance of these free cysteines to activity was shown by chemical modification studies, in which two of the three free cysteines need to be in the properly reduced state to preserve catalytic functions and prevent aggregation (T Edmunds, pers. commun.). Alteration of this reduction state leads to irreversible losses of activity due with exposure to pH > 7.0. The wild-type human enzyme has high sensitivity to pH > 7.0, with a $t_{½}$ of ~ 5 min for activity. This inactivation is irreversible and has implications for enzyme therapy. A significant amount of work has been accomplished with site-directed mutagenesis to prove the causality of mutations related to Gaucher disease [44]. Of importance to enzyme therapy, two different variants of the GCase have been available for clinical use: the enzyme purified from human placenta and the human GCase produced in Chinese hamster ovary (CHO) cells. The amino acid sequences of these proteins are identical except for a R495H substitution in the recombinant GCase, compared with the placental form [48,49]. Site-directed mutagenesis and expression studies show that this substitution has no effect on the properties of the enzyme [50]. Indeed, the kinetic properties and cellular uptake, as well as the therapeutic efficacy and safety of the two enzyme variants are essentially identical [50,51].

4. Delivery of β-glucosidase to tissues

As indicated above, the primary target site of pathology and for therapy in Gaucher disease Type 1 is the macrophage, or macrophage precursors. The accumulated lipid, glucosyl ceramide within these cells leads to the production of cytokines and chemokines that propagate the disease manifestations. The macrophage has a specific receptor on its surface, the MMR, for the uptake of α-mannosyl-terminated oligosaccharides and glycoproteins [33,52]. This receptor participates in the innate immunity response against specific fungal infections whose antigens contain large arborising mannan structures [33,52].

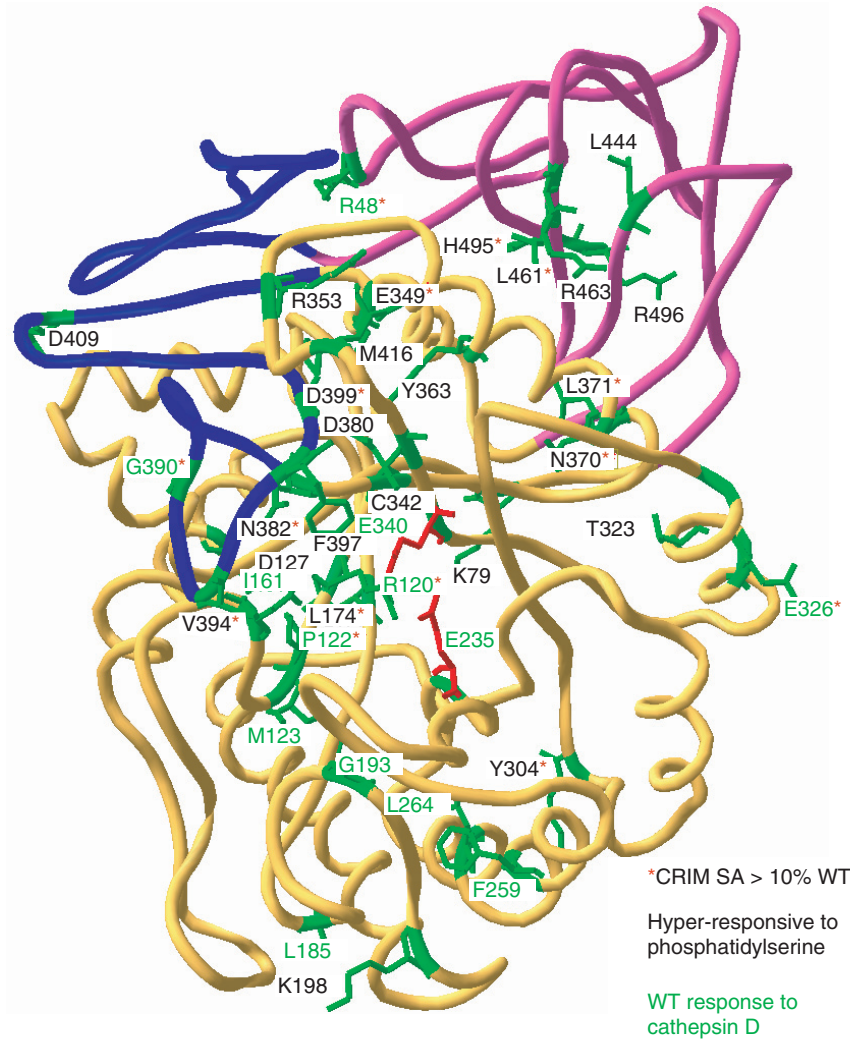

Figure 4. Representation of the crystal structure of human GCase. Several amino acid residues that are mutated in Gaucher disease patients are indicated by the wild-type amino acid residue. The bar structures in represent E340 and E235 that are the active site nucleophile and acid/base involved in catalysis. Green residues indicate mutated sites that produce enzymes with wild-type stability to cathepsin D digestion *in vitro*. Residues (*) indicate mutated enzymes that retain ≥ 10% of wild-type K_{cat}. The three domains are indicated in blue (X domain), yellow (catalytic domain) and red (IgG-like domain).
CRIM: Catalytic rate constant based on crossreacting immunological material; GCase: β-Glucosidase; SA: Specific activity; WT: Wild-type.

As shown in **Figure 5**, MMR has a structure composed of eight carbohydrate recognition domains that have specificity for α-mannosyl residues. Three or four of these have activity and participate in the high affinity binding to terminal α-mannosyl residues. Although human GCase is known to contain complex carbohydrate modifications, the core consists of α-mannosyl residues (**Figure 3**). GCases from placenta and CHO cells have been modified to expose such residues by sequential enzymatic digestion to remove terminal sialic acid, galactose and *N*-acetyl-glucosamine residues, and exposure of the α-mannosyl core structures. This modification produces placental or recombinant GCases with very similar uptake and distribution properties [51] (e.g., the K_d values MMR on rat alveolar macrophages were 13.85 ± 3.95 and 12.45 ± 3.45 nM for the CHO and placental derived enzymes, respectively). An alternative method to produce α-mannosyl-terminated GCase is to use inhibitors of α-mannosidase I (kifunensin; **Figure 1**). This would leave oligosaccharides with ~ 9 α-mannosyl residues for use in uptake by the MMR. The effect of this approach on differential uptake or survival of the GCase is not known, but the use of a mannosidase I inhibitor could lead to high-mannose containing oligosaccharide side chains that would be accessible for the mannose-6-phosphate modification enzymes. This potentially could redirect the GCase away from the MMR to the more ubiquitous mannose-6-phosphate receptors. Structural and functional studies are needed to evaluate this potential. Imiglucerase from CHO cells has been shown to be highly effective in the treatment of Gaucher

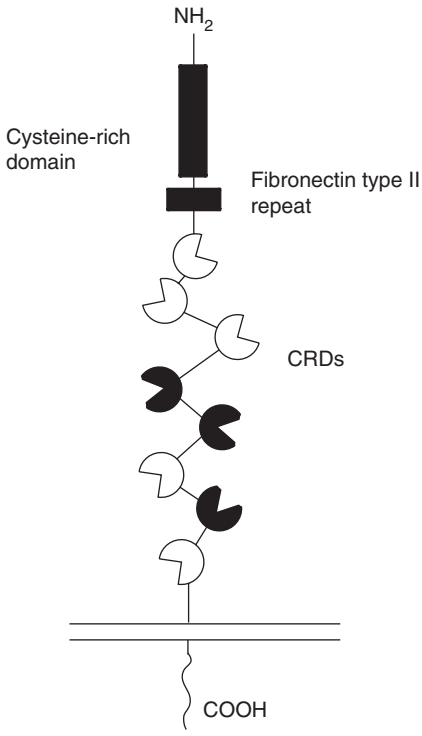

Figure 5. Schematic of the MMR. The carbohydrate recognition domains with or without shading indicate binding or non-binding subsites. The NH_2 and COOH termini are on the exterior and cytoplasmic surfaces of the macrophages. Once α-mannosyl residues are bound, the receptor-ligand complex is endoytosed for delivery to the lysosome.
CRD: Carbohydrate recognition domain; MMR: Macrophage mannose receptor.

disease [53-55]. GCase derived from the mannosidase I inhibitors are currently under investigation by TKT/Shire. Also, recent data suggest a downregulation of the MMR at particular stages of Gaucher cell development [9]. This has clear implications for therapy and therapeutic efficiencies.

The selective targeting of exogenous enzymes to inappropriate tissues and intracellular sites could have significant therapeutic implications through at least two mechanisms: i) expansion of the distribution space of the enzyme to include pathogenically uninvolved tissues, and ii) redirection of enzyme away from target sites of pathology. For example, the development of non-inhibitory antibodies to administered enzymes for any of the lysosomal diseases could lead to the enzyme–antibody complexes and direction of these to Fc receptors on macrophages or other cell types and away from involved tissues (i.e., endothelial cells or muscle). Such a mechanism could account for the localisation of the majority of intravenously administered lysosomes being targeted to the hepatocytes through the asialoglycoprotein receptor system. Similar redistribution could operate at a subcellular level and redirect enzyme away from lysosomes. Both mechanisms could lead to diminished therapeutic and clinical reponsiveness.

5. Intravenous/parenteral delivery of β-glucosidase

Available published data relate to the placental (alglucerase) or CHO-cell-derived (imiglucerase) human GCases. Essentially, all pharmacodynamic/kinetic data have been obtained in wild-type rodents, because, until recently, no viable mouse models of Gaucher disease were available [56]. In addition, all studies have used bolus intravenous injections. Upon tail vein bolus injection into mice of non-saturating amounts of mannose-terminated GCases, the plasma disappearance $t_{½}$ values vary from 1.5 to 7.0 min [51,57-59]. At greater than saturating bolus doses, the $t_{½}$ for plasma disappearance was ~ 20 min [59]. Uptake of these enzymes was primarily (66 – 95%) into the liver, with smaller amounts being taken up into cells of the spleen, lung and kidney. Recoveries of activity were ~ 50% of the total administered activity. No distribution of the enzyme into the CNS was detected when the mice were perfused with saline to eliminate the enzyme remaining plasma. Following intravenous bolus injections, the disappearance $t_{½}$ values of recovered GCase in cells of several tissues were biphasic [51,57-59]. The first phase was rapid, with a $t_{½}$ of ~ 1 h. The second phase ranged from $t_{½}$ ~ 12 – 14 to 48 – 72 h for both activity and protein. The reasons for the significant differences between the second phase results are not apparent from the experimental details. The ~ 50% recovery of enzymatic activity was due, in part, to GCase denaturation, as, based on quantitation of the amount of enzyme protein, GCase activity per molecule was only ~ 50 – 70% of preinfusion activity (i.e., the enzyme was denatured to a significant extent). In addition, the enzyme does not regain activity upon entry into cells or to the lysosomes following such bolus injections [59].

The delivery of enzyme to the liver was into hepatocytes (65 – 90%), Kupffer cells (5 – 15%) and endothelial cells (10 – 20%), with varying percentage recoveries in different studies. One study found that the primary site of localisation to the sinusoidal endothelial cells [58], whereas the other study showed the primary uptake was into hepatocytes [51]. Both studies showed significant concentrations in Kupffer cells but this accounted for ~ 10 – 15% of the total enzyme recoverable from the liver. Thus, delivery of injected GCase to livers is to all cell types, but with significant concentrations in Kupffer cells. In a head-to-head comparison, recombinant enzyme had greater concentrations in Kupffer cells (~ 22%) compared with that in placental derived enzyme (11%), and the opposite for sinusoidal endothelial cells (3 and 11%, respectively). The subcellular localisation was shown to be to the lysosomes [51].

Recently, a different lysosomal enzyme, lysosomal acid lipase, was purified from either tobacco plants or Pichia pastoris. Both sources provide enzymes with terminal mannosyl residues. In the background of an absent MMR, these enzymes were distributed to hepatocytes, endothelial cells, and, surprisingly, to Kupffer cells, with significant therapeutic benefit in the lysosomal-acid-lipase-deficient mouse model [60]. Thus, several

Table 1. Therapeutic goals for gaucher disease type 1.

Parameter	Goal	Time to goal
Anaemia	Improve and maintain haemoglobin at normal levels (age-, sex-dependent levels)	12 – 24 months
Thrombocytopenia	Increase and maintain platelet count sufficient to avoid bleeding difficulties: i) Splenectomised patients – normalise ii) Intact spleen – increase 1.5- to 2.0-fold and then to low normal iii) Avoid splenectomy	i) 12 months ii) 12 – 24 months
Hepatomegaly	Decrease and maintain liver volumes at 1.0 – 1.25 normal volumes: i) Decrease by 20 – 30% ii) Decrease by 30 – 40%	i) 12 – 25 months ii) ~ 36 months
Splenomegaly	Decrease and maintain spleen volume < 2 – 8 normal volumes: i) Decrease by 30 – 50% ii) Decrease by 50 – 60%	i) 12 months, ii) ~ 24 – 36 months
Bone	i) Lessen or eliminate bone pain ii) Prevent bone crises iii) Attain ideal peak bone mass in children	i) 12 – 24 months ii) 12 – 24 months iii) By puberty
Paediatric growth	i) Achieve normal growth rate ii) Achieve normal puberty	i) By 36 months ii) Family adjusted
Pulmonary involvement	Reverse hepatopulmonary syndrome, decrease/eliminate pulmonary hypertension, prevent pulmonary failure	To be developed
Quality of life	i) Restore normal daily activities, ii) Improve quality of life scores on validated tests	i) Patients adjusted ii) 24 – 36 months

receptors may be participating in the uptake of these mannosyl-terminated enzymes in addition to the MMR. Alternatively, the small amount of β-galactosyl residues on oligosaccharides from these sources may have significant ability to redirect the enzymes via their specific receptors.

In two human studies in patients with Gaucher disease, a similar pattern of uptake was shown in liver or spleen [61,62]. In liver, sinusoidal lining cells (endothelial and Kupffer cells) showed significant uptake of imiglucerase. The spleen showed greatest uptake in macrophages [62]. There are no other detailed human studies available. No studies have been published in a mouse model of Gaucher disease. Importantly, in all studies, no distribution of the enzyme into CNS could be detected following intravenous infusions.

6. Clinical effects of intravenous infusions in humans

Regular intravenous infusions of recombinant or placental derived GCase has led to improvements in liver, spleen, haematological, bony and other visceral manifestations of patients affected with Gaucher disease [53-55,63-65]. The viscera have been significantly improved in all the variants, but no significant effect has been demonstrated on the CNS manifestations in the neuronopathic variants (types 2 and 3). Importantly, the visceral responses are slow (i.e., months to years are required for observable specific improvements in various organ systems). The most rapid improvements are in the liver and splenic volumes, but decrease significantly by 6 – 12 months [54,65]. The haematological parameters, particularly anaemia, thrombocytopenia and leukopenia, show significant improvement in the first 12 months. Based upon experience in > 2000 patients, therapeutic guidelines and milestones with goals and timeframes for achievement have been developed for monitoring the responses to regular intravenous GCase infusions [65]. These are summarised in **Table 1**. In general, within ~ 1 year, the hepatic, splenic and haematological abnormalities should show substantial improvement, whereas improvement in reversible bony disease occurs only within ~ 3 – 5 years. A substantial controversy has surrounded the selection of optimal dosing, particularly in view of the great cost of imiglucerase, (US$4/U/kg body weight every 2 weeks [i.e., for a 70-kg adult at 30 – 60 U/kg, the cost is US$8400 –16,800 every 2 weeks]). This controversy has been based primarily on personal/social arguments. Both have some merit for those of us who wish to provide the best care delivery to Gaucher disease patients. Informed decisions should be based on data/evidence, and, in rare diseases, such data require significant time to acquire and are just now emerging. A preliminary report of 366 patients using an analysis based on phenotype matching indicates a significant positive relationship between dose and hepatic and splenic volume decreases, as well as to increases in haemoglobin and platelets [66]. Of importance, both the initial response rate and the long-term (> 3 years) sustained response also correlated

directly with doses of 15, 30 or 60 U/kg every 2 weeks. More complete analyses will be needed to provide data for optimising/individualising enzyme therapy for each patient, but such analyses provide a consistent methodology for analysis.

Among adverse events that occur in ~ 15% of patients, allergic symptomatology predominates: including urticaria, pruritus, nausea, vomiting and occasional palpitations [54,67,68]. The majority of these adverse events are treatable by antihistamines and rarely need corticosteroids. Approximately 50% of these adverse events (~ 7.5% of treated patients) are IgG antibody mediated [68]. Patients become tolerised to the administered protein within 24 – 36 months of continuous therapy [68]. Approximately five IgE-mediated adverse events are known from the > 4200 patients worldwide on enzyme therapy. Also, ~ 12 – 13% of patients receiving either alglucerase or imiglucerase develop antibodies to the proteins [68,69] (i.e., there is no difference in antibody positivity between the preparations) [70]. This is in contrast to the 50 – 90% antibody positivity rate in patients receiving agalsidase α or β [4,71], laronidase [3] or alglucosidase α [5,72,73] for Fabry disease, MPS I, or Pompe disease, respectively. This difference in antibody development rate may be due to the intrinsically lower antigenicity of GCase, but this has not been proved. In addition, four cases of inhibitory antibodies to GCase are known, with *in vivo* effects leading to worsening of the disease in patients [74,75]. These patients have been tolerised using very high doses of enzyme therapy [69].

7. Other delivery methods

7.1 Gene therapy approaches

Bone marrow transplantation (BMT) to replace defective macrophages with macrophages containing fully functional GCase has been used to treat Gaucher disease type 1 [76-81]. However, the high morbidity and mortality of BMT and the safety of enzyme therapy has led to the latter becoming the standard of care. The BMT experience as a curative strategy suggests that stem cell transplantation would be a viable strategy using GCase gene corrected/supplemented cells from individual patients. In mice and humans, retroviral-mediated gene transfer into haematopoietic stem cells has lead to persistence of the transgene and expression of GCase to levels that could potentially be therapeutic [82-84]. In addition, this approach has the possibility of supplying corrected microglial cells to the CNS from the differentiation of bone-marrow-derived monocytes. However, unlike MPS I and several other lysosomal storage diseases, BMT was unable to prevent development of CNS disease in a Gaucher disease type 3 patient who was neurologically intact prior to the uncomplicated BMT [61].

Proof-of-principle has been obtained for the organoid approach for gene therapy of Gaucher disease in mice. A single intravenous injection of adeno-associated virus serotype 8, containing the human GCase cDNA into a Gaucher mouse model, led to long-term (~ 1.5 years) expression in the liver and secretion of GCase at very high levels into the serum [82]. Correction of histological and/or biochemical abnormalities was documented in the liver and spleen, and, partial correction in the lungs. A similar approach used a conditional expression system based on the tetracycline transactivating system to express GCase in liver. In the presence of tetracycline, the human enzyme expression was turned off. In a Gaucher mouse model, large amounts of GCase were produced in the liver and secreted into the plasma (~ 100 μg/ml). This enzyme was delivered to other tissues, including the spleen and lungs, with substantial therapeutic effect (Sun and Grabowski, unpublished). Upon introduction of tetracycline to turn off the enzyme expression for 2 months, the storage cells and substrate accumulation returned. The carbohydrate composition of the liver-produced enzyme was not characterised, but, by immunoblotting patterns, complex carbohydrate modifications were suggested. This modification would probably lead to inefficient delivery to specific macrophages. Thus, although the organoid approach could be used and could be considered in patients with Gaucher disease, the method appears to be relatively inefficient for the targeted delivery of enzyme. In addition, the large amounts of enzyme delivered to specific tissues may lead to an imbalance in the stoichiometry between GCase and saposin C, further diminishing the effectiveness of this approach.

None of these approaches provide delivery of enzyme to the CNS. It is quite clear that any delivery route based on intravenous administration, either endogenously or exogenously, will not provide for delivery of GCase to the brain by crossing the blood–brain barrier. Studies are underway to evaluate the use of direct injection of GCase into the ventricles for distribution throughout the CNS. However, these will necessarily be based on the stage of the CNS disease, as the primary abnormality in the CNS is not high-level storage of glucosyl ceramide, but rather neuronal death.

7.2 Pharmacological chaperones

Pharmacological chaperones, using reversible competitive inhibitors of GCase, provide additional interesting drug delivery systems for Gaucher disease. The current focus on pharmacological chaperones has been on the ability of selected inhibitors to redirect or to direct poorly targeted mutant enzymes away from the ER and Golgi to the lysosome by reconforming them around the inhibitor [85-87]. Although this retargeting and delivery of newly synthesised protein to the lysosome may be one part of its mechanism of effect, clearly the mechanisms are more complicated and may include the GCase within the lysosome being reconformed for greater activity. Moreover, the mutation specificity for particular inhibitors to have a chaperone effect will need to be fully evaluated. Thus, although the mechanisms are not clear, they show some promise for enhancement of activity, but certainly not to the levels that can be obtained by infusions of enzyme. Their clinical efficacy has yet to be shown in either model or human systems.

8. Expert opinion

The parenteral administration of GCase by intravenous routes for the treatment of Gaucher disease will have a finite lifespan. The utility of enzyme therapy for the treatment of Gaucher disease is undeniable, as it improves to varying degrees all the major aspects of the visceral disease in Gaucher, if instituted at the proper time. The current system of intravenous injections every 2 weeks is both inconvenient and inefficient. The inefficiency derives not only from the medical care delivery system, but from the basic enzyme properties leading to denaturation and losses of activity during the complex mechanism of delivery of GCase from the circulation into the lysosome. Therefore, other routes will need to be explored for convenience. Intramuscular, inhaled or depo-versions might be considered, although will not be realistic alternatives in the near future. Oral administration of enzyme seems highly unlikely unless a method is developed to protect the enzyme from digestion within the gastrointestinal tract. Such methods may also permit a more sustained, or, at least, less frequent delivery of GCase to tissues that could be more efficient at a cellular level by preventing/minimising interdose reaccumulation of glucosylceramide. Combination therapies with pharmacological chaperones could avoid some of the denaturation/destabilisation effects of hostile environments.

Some consideration has been given to reengineering GCase into a more active or better molecule, potentially in combination with saposin C. The interaction of GCase with saposin C appears to be complex and involves not only interaction of the two proteins, but also some reconformation of membranes. Thus, it is unlikely that this combination would be of significant benefit and would add complexity to an already cumbersome treatment schedule. Moreover, the reengineering of GCase into a more active form might prove to be very difficult. Throughout phylogeny, GCase is retained with high conservation. In mammalian species, the amino acid sequences of GCase are 80 – 90% amino-acid identical. A 50% amino acid identity occurs between human and Fugu (the last common vertebrate ancestor), and ~ 35 – 40% identity between man and worm. Thus, the enzyme has very little flexibility in its ability to be modified to alter activity. However, based on the crystal structures of human GCase, some reengineering may become evident, but with obvious potential antigenic concerns.

Alternative production systems must be considered for GCase. A variety of alternative mammalian cells (e.g., CHO and human skin fibroblasts) have been tried, and the differences in therapeutic efficacy are likely to be small. Plant-produced GCase may have significant promise for the large scale production of mannose-terminated enzymes, but the presence of a core xylose in plants compared with a core fucose in mammals on the *N*-acetyl-glucosamine of the oligosaccharides chain may lead to antigenic problems of these proteins made in plants. GCase produced in yeasts with large arborised mannan oligosaccharide chains would not be of much utility, given the antigenicity of these sugars. Such modification may also lead to redistribution of the enzymes to cell types, with little or no contribution to the disease itself. However, efficient production or convenient delivery systems will only incrementally alter the utility of therapy, and likely not have significant impact on cost, efficacy or convenience of a lifelong treatment.

The pharmacological chaperones appear to have a significant possibility for therapeutic effect. The major conceptual concern is the delineation of the intralysosomal level of residual enzyme activity necessary and sufficient for therapeutic effect. Although large-fold changes in enzyme activity have been found using *in vitro* assays for this approach, the absolute amount of enzymatic activity may not exceed a threshold for therapeutic utility. Furthermore, active-site directed pharmacological chaperones have the potential to accentuate the disease process (i.e., too great a concentration of the chaperone could inhibit the endogenous enzyme to very low levels). This concern may be greatest with patients in whom the residual GCase activity is at or near a threshold for a categorical change in phenotype (e.g., the border between types 2 and 3, or types 1 and 3). Model organism studies are clearly needed to address these concerns.

Also evident is the incompleteness of monotherapy for the lysosomal storage diseases. The combination of developing enzyme, gene, substrate reduction and pharmacological chaperone strategies will increase the overall effectiveness by exploiting the strengths (potentials) of each, including visceral efficacy, continuous expression, attenuation of redevelopment, and intracellular engineering and tissue distribution. This paradigm shift, coupled with validated clinical parameters of and biomarkers for assessing disease burden, should improve the overall cost-effectiveness of therapy by personalising care plans to individual patient needs.

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