Chemical biology of glucosylceramide metabolism fundamental studies and applications for Gaucher disease

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Citation
Oussoren, S. V. (2017, September 28). *Chemical biology of glucosylceramide metabolism fundamental studies and applications for Gaucher disease*. Retrieved from https://hdl.handle.net/1887/55842

Cover Page

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Discussion

Discussion

GBA in health and disease.

Glucocerebrosidase (GBA: EC 3.2.1.45) is the lysosomal β-glucosidase degrading glucosylceramide (GlcCer) to ceramide, an essential step in the cellular recycling of glycosphingolipids[1,2]. The enzyme, a 495 AA glycoprotein with 4 N-linked glycans, employs for catalysis a double displacement mechanism with glutamate 340 as nucleophile and glutamate 325 as acid/base residue[3]. GBA (also named GBA1), unlike other soluble acid hydrolases, is not sorted to lysosomes via mannose-6-phosphate receptors, but through binding to the lysosomal integral membrane protein II (LIMP-2)[1] (figure 1). Deficiency of GBA forms the molecular basis of Gaucher disease (GD), a recessively inherited lysosomal storage disorder[2]. GD patients have two mutated GBA alleles encoding mutant forms of GBA. Most of these mutations encode single amino acid substitutions that may impair folding of the enzyme in the endoplasmic reticulum and/or stability and catalytic activity in lysosomes[2]. A significantly reduced GBA activity causes ongoing accumulation of GlcCer in storage deposits inside lysosomes, a phenomenon that particularly occurs in macrophages of GD patients[2]. Most cell types of GD patients manage to limit lysosomal glucosylceramide accumulation through its conversion to glucosylsphingosine, the sphingoid base that may leave lysosomes and even cells[1].

Chronic supplementation of the lysosomes of macrophages with normal (recombinant produced) GBA forms the basis for the registered enzyme replacement therapy (ERT) of GD[4–6]. This treatment, employing enzyme with mannose-terminated N-linked glycans favoring uptake by macrophages, is remarkably effective in non-neuronopathic type 1 GD patients: it results in prominent reversal and prevention of hepatomegaly, splenomegaly and hematological abnormalities and thus markedly improves the quality of life of patients[6]. ERT is now worldwide applied for over 25 years in type 1 GD patients with a non-neuropathic disease course. The long-term efficacy of ERT has meanwhile been thoroughly analyzed[7]. The various recombinant enzyme preparations nowadays used for ERT are relatively expensive and largely determine the cost of the treatment, usually ranging from €100.000 to 300.000 per year for an adult patient[8]. Individualized dosing regimens are increasingly used to reach optimal clinical response at a minimum of costs[5]. Biomarkers, ranging from Gaucher cell-derived proteins (chitotriosidase, CCL18, soluble gp-NMB) to lipids (glucosylsphingosine), are increasingly used to monitor response to ERT and optimize treatment regimens[1,9]. Present ERTs with macrophage-targeted recombinant enzymes unfortunately do not prevent neurological manifestations in the more severely affected (type 2 and 3) GD patients. ERT neither offers full protection against skeletal disease, pulmonary hypertension, gammopathies and related leukemia's and Parkinson's disease[7].

The considerable costs of the present ERTs and moreover the clinical limitations of the treatment motivate ongoing investigations on additional therapies for GD (table 1), including the research described in this thesis that focused on elucidating factors modulating lysosomal GBA (see figure 2). The identification of GBA deficiency as major risk factor for developing α-synucleinopathies has boosted further the research interest in GBA and its therapeutic modulation[10].

Modulating lysosomal GBA.

The initial focus of research described in this thesis originates from the observation in the eighties that exposure of cells to leupeptin, a serine and cysteine protease inhibitor[11], causes an increase in GBA. Compared to other lysosomal hydrolases, GBA survives relatively short in lysosomes due to fast proteolytic degradation. This is even more pronounced for some mutant forms of GBA occurring in GD patients, for example N370S GBA that is present in the vast majority of Caucasian type 1 GD patients and shows slightly abnormal kinetic parameters[11–15]. Like wildtype GBA, the mutant N370S enzyme folds in the endoplasmic reticulum and subsequently ends up in lysosomes (figure 1). However, inside lysosomes N370S GBA undergoes abnormal fast proteolytic degradation, a process that can be inhibited by leupeptin[11]. Thus, pharmacological augmentation of mutant GBA's like the common N370S enzyme through inhibition of their lysosomal proteolytic degradation offers in theory a novel therapeutic avenue to treat specific GD patients (figure 2).

At the start of this thesis research (chapter 1), the old finding that cellular GBA is significantly increased by leupeptin was first re-capitulated. Subsequently, it was established that stabilization of the enzyme can also be induced by E64 (IUPAC name: (1*S*,2*S*)-2-(((*S*)-1-((4-Guanidinobutyl)amino)-4-methyl-1-oxopentan-2-yl)carbamoyl)cyclopropanecarboxylic acid), an irreversible inhibitor of cysteine proteases, but not by AEBSF (IUPAC name: 4-(2-Aminoethyl)benzene sulfonyl fluoride), an inhibitor of serine proteases (data not shown). E64 has been earlier used by Greenbaum *et al.* as scaffold of an activity based probe (ABP) named DCG-04[16]. This ABP when tagged with a fluorescent BODIPY group (DCG-04 BODIPY) covalently binds to the cathepsins C, S, F, H, K, V, B and L, each proteases with a catalytic mechanism involving a nucleophilic cysteine thiol in a catalytic triad or diad[17]. Labeling of GBA with a specific BODIPY tagged cyclophellitol-epoxide ABP[18] and labeling of proteases with DCG-04 BODIPY in combination with electron microscopy (correlative light and electron microscopy imaging) confirmed co- localization in lysosome structures (DM van Elsland, unpublished data). It was next established that BODIPY DCG04, like leupeptin and E64, stabilizes GBA in cultured cells. Exposure of cultured lymphoblasts from N370S GBA carrying GD patients was found to reduce their glucosylsphingosine content, a sign of functional correction of lysosomal GlcCer metabolism[1,19]. The cathepsins C, S, F, H, K, V, B and L were all candidates to mediate the lysosomal degradation of GBA. It was therefore next investigated which of the cathepsins mediate the breakdown of GBA. For this purpose, specific cathepsins were deleted by shRNA and/or CRISPR-CAS technology (chapter 1). Moreover, *in vitro* digestion of recombinant GBA by commercially obtained recombinant cathepsins B, F, H, L and S was examined. The combined findings led to the conclusion that not a single cathepsin is entirely responsible for lysosomal degradation of GBA. Apparently there exists redundancy among the examined cysteine cathepsins in degrading GBA, as illustrated by the observed

compensation of each's absence. In other words, to slow down intra-lysosomal proteolytic turnover of GBA multiple cathepsins will need to be concomitantly inhibited. Such broad inhibition likely results in major impairment of protein turnover in lysosomes in general and a chronic intervention along this line therefore offers no realistic therapy.

Since a therapy based on specific inhibition of the proteolytic degradation of GBA inside lysosomes seems elusive, attention was next focused to the structural stability of the enzyme inside lysosomes (figure 2). Key questions to be addressed in this connection are which factors stabilize the fold of GBA in lysosomes and does re-folding occur? The structural stability of recombinantly produced GBA was first examined (chapter 2). In the experiments use was made of known reversible inhibitors as well as cyclophellitol-based ABPs that covalently bind to the catalytic nucleophile glutamate 340[3]. It was found that occupancy of the catalytic pocket with inhibitors stabilizes the fold of the enzyme. The melting temperature (T_m) of GBA determined by tryptophan fluorescence and circular dichroism is increased by the presence of inhibitors interacting with the catalytic pocket. The effect is smallest for hydrophilic reversible inhibitors like isofagomine, more potent with the semi-reversible 2-deoxy-2-fluoro-β-d-glucopyranosyl-2-deoxy-2-fluoro-β-d-glucopyranosyl-N-phenyltrifluoroacetimidate and highest with irreversible cyclophellitol-based inhibitors tagged with a hydrophobic BODIPY moiety[18]. Similar, GBA proved more resistant against trypsin digestion upon prior incubation with irreversible inhibitors, again particularly with the amphiphilic cyclophellitol-type ABPs. In summary, it is quite apparent that structural stability of GBA is remarkably improved by the occupancy of the catalytic pocket with an amphiphilic inhibitor. Consistent with this conclusion are the observations that an ABP pre-labeled GBA after uptake by cultured macrophages is remarkably protected against proteolytic degradation and the same holds for pre-labeled enzyme following infusion in mice (chapter 2,[20]).

The beneficial outcome of occupancy of the catalytic pocket with inhibitors regarding structural stability of GBA and its resistance against proteolytic breakdown are of great interest. It might be speculated that the physiological substrate glucosylceramide exerts a similar protective effect. In such case, the lack of substrate would promote the degradation of "idle" enzyme. Another implication would be that lysosomes will tend to always contain a small residual amount of glucosylceramide.
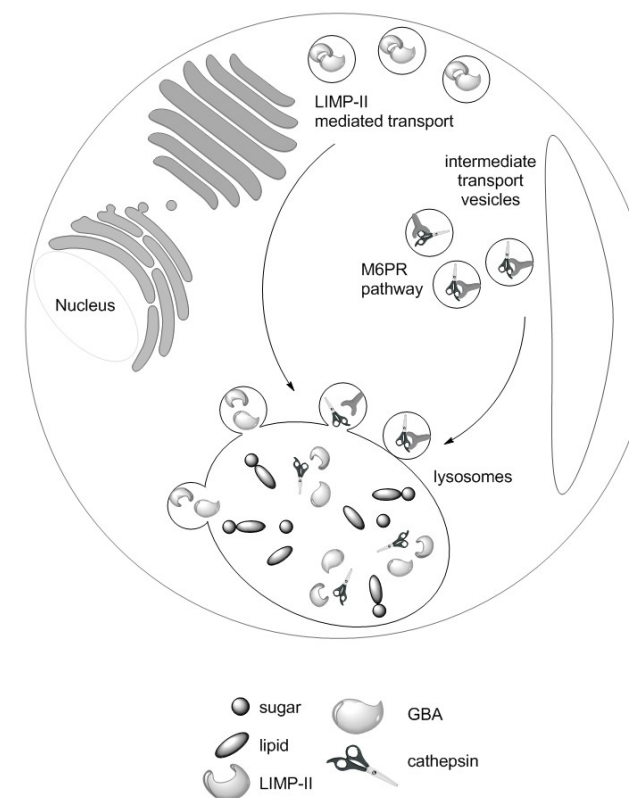

Figure 1. Cellular pathways of GBA via LIMP-2 associated transport and other lysosomal proteins such as cathepsins via M6P-mediated transport. LIMP-II: LIMP-2.

Catalysis by GBA.

The catalytic pocket of GBA, in particular the glycon binding domain, can nowadays be probed with cyclophellitol-type ABPs designed by researchers at the Leiden Institute of Chemistry at Leiden University and Academic Medical Center of the University of Amsterdam[18,21]. In sharp contrast, insight in the aglycon (lipid) binding domain of the enzyme is at present still very limited which seriously impairs full understanding of the actions of the enzyme. The relevance of the lipid moiety binding (aglycon) domain is highlighted by the recent realization that GBA can act as transglycosylase[22]. When exposed to a high concentration of cholesterol, GBA may transfer the glucose from a β-glucoside substrate to the sterol and thus generate glucosyl-β-cholesterol (GlcChol)[22]. The physiological relevance of this reaction is illustrated by the accumulation of GlcChol in patients suffering from Niemann-Pick type C disease (NPC). This lysosomal storage disorder is due to an inherited deficiency in egress of cholesterol from lysosomes and leads to dramatic lysosomal sterol accumulation. U18666A-induced lysosomal cholesterol accumulation also promotes GlcChol generation in cultured cells[22]. More recently it was noted that GBA, *in vitro* as well as in cultured cells, when exposed to β-xyloside

substrates acts as β-xylosidase and transxylosylase generating xylosylated sterols (chapter 4). The physiological relevance of these activities of GBA towards β-xylosides still needs to be established. In particular, it will be of interest to study the natural occurrence of xylosylated sphingolipids and sterols.

The observed formation of GlcChol by GBA suggests that the enzyme has a high affinity binding site for sterols in its catalytic region. To further increase insight in this matter, a new procedure to probe the catalytic pocket of GBA with a lipid substrate was developed (chapter 3). A mimic of glucosylceramide equipped with an internal diazirine and terminal alkyne in its fatty acyl moiety, so-called pacGlcCer, is used for this[23]. Following incubation of GBA with pacGlcCer, the diazirine in the lipid can be UV cross-linked to closely neighboring amine groups of the protein. Hereafter, a fluorophore can be linked to the alkyne by click chemistry allowing the detection of lipid-labeled protein following SDS-polyacrylamide gel electrophoresis. Using this method, it was observed that saturated labeling of enzyme with pacGlcCer already occurs at low lipid concentration, pointing to a high affinity interaction of pacGlcCer with GBA. This is not surprising since pacGlcCer proves to be an excellent substrate for GBA. The binding of pacGlcCer to GBA shows similar pH dependency as the enzymatic activity, again suggesting that the lipid preferentially binds in the catalytic pocket like the natural glucosylceramide substrate. Next it was noted that compounds binding in the glycon binding site of GBA like cyclophellitols prohibit pacGlcCer labeling of the enzyme. This further confirms that pacGlcCer is truly bound in the catalytic pocket. Of interest is the finding that the presence of cholesterol, an acceptor in transglycosylation, prohibits already at low concentration the labeling of GBA with pacGlcCer. Thus, the high affinity cholesterol binding site must be part of the catalytic pocket. In principle, the new method, in conjunction with the ABPs, can be used to probe which compounds interact with high affinity with the catalytic pocket of GBA, both the glycon and aglycon binding domain.

The novel findings presented in Chapters 3 and 4 illustrate that GBA is catalytically far more diverse than hitherto considered. The physiological relevance of the noted transglycosylase and β-xylosidase capacity of GBA warrants further research. In the first place, the impact of Gaucher disease-associated mutations in GBA on various catalytic activities needs to be investigated. Of further interest in this connection is the occurrence of active GBA in the extracellular layer of the skin, the stratum corneum[24]. This membrane layer is very rich in glucosylceramide and cholesterol and it is theoretically conceivable that GBA might locally generate GlcChol. Another consideration worthwhile to explore further is the potential use of β-xylosides as stabilizing chaperones for GBA. In principle, carefully selected β-xylosides might also stabilize GBA in its structure and render protection against proteolytic breakdown. Such β-xylosides are likely relative poor substrates compared to β-glucosides and therefore might act as long-lived stabilizers inside lysosomes.

Proteins influencing GBA inside lysosomes.

The sorting of newly formed GBA to lysosomes has been enigmatic for a long time. It was soon clear that GBA acquires no mannose-6-phosphate moieties in its glycans and therefore is not transported to lysosomes through binding to mannose-6-phosphate specific receptors[1,25]. A major breakthrough was the discovery that the lysosomal integral membrane protein 2 (LIMP-2) following binding of GBA in the ER, transports the enzyme to lysosomes[26,27,28]. Although low pH is reported to promote dissociation of GBA and LIMP-2, it seems likely that interaction of the two proteins still (transiently) occurs in lysosomes[10]. Indeed, the experiments described in Chapter 5 provide evidence that LIMP-2 protects the enzyme against proteolytic degradation in lysosomes. HEK293T cells expressing mannose receptors were found to endocytose well *Cerezyme* (mannose-terminated recombinant human GBA). The half-life of recombinant GBA was considerably shorter in cells lacking LIMP-2 as the result of faster proteolytic degradation. The stabilizing effect of the interaction of GBA with LIMP-2 in lysosomes might be of great importance regarding ERT. It seems conceivable that LIMP-2 becomes "rate limiting" during ERT with high doses of therapeutic enzyme. Administration of very high doses of *Cerezyme* might result in lysosomal enzyme concentrations that exceed the capacity of LIMP-2 to stabilize it. In view of this consideration of the recent finding that a LIMP-2 helix 5-derived peptide is able to activate GBA is highly relevant[29]. Co-administration of a stabilizing LIMP-2 fragment and recombinant enzyme might be considered for ERT. In addition, the LIMP-2 peptide might be able to increase endogenous GBA capacity in those GD patients with residual lysosomal enzyme.

Saposin C (SapC) is another protein known to interact with GBA inside lysosomes[30]. SapC is formed by proteolytic cleavage from the large precursor prosaposin[31,32]. It is an 80 amino acid peptide that binds in nanomolar range to GBA and thus stimulates the enzyme in activity towards natural lipid substrate GlcCer as well as artificial (water-soluble) substrates, β-glucosides and β-xylosides alike (chapter 4)[33–36]. The interaction of SapC with GBA is strongly promoted by the presence of negatively charged phospholipids, in particular the lysosomal phospholipid BMP (bis(monoacylglycero)phosphate)[36,37]. The physiological relevance of the activation of GBA by SapC is illustrated by the finding that deficiency of SapC causes GD-like pathology, including the presence of Gaucher cells and related markers[38–40]. Over the years several hypotheses have been put forward regarding the physiological action of SapC. Initially a role for SapC in facilitating access of lipid substrate to the pocket of GBA was envisioned as its primary mode of action[41]. According to this mechanism, SapC would solubilize GlcCer from membranes allowing its hydrolysis by GBA to take place in an aqueous environment [42,43]. Evidence for a direct interaction of SapC with membranes has indeed been presented. SapC was shown to destabilize membranes and promote fusion[44–46]. Binding of SapC to GBA affects the enzyme as such. Clearly, SapC binding influences the catalytic pocket of GBA, as reflected by increased catalytic activity and changed substrate affinity. It remains unclear whether SapC also promotes structural stability of GBA. SapC has been proposed by Sun *et al*. to protect GBA from proteolytic degradation by facilitating association of GBA with membranes[47]. Moreover, it was speculated that specific mutations in GBA reduce the interaction with SapC and thus cause a decreased stability of the enzyme[47]. In view of these observations, the shortened lysosomal half-life of N370S GBA might partly be caused by diminished stabilization by SapC. The residues 48-122 of GBA appear to be involved in the activation of enzyme by SapC as

established by effects on enzymatic activity[48]. A docking model of GBA-SapC interactions generated by Atrian *et al.* suggests that the SapC binding sites in GBA are the TIM barrel-helixes 6 and 7 and the Ig-like domain[49]. Further studies, in particular co-crystallization of GBA and SapC, are required to precisely establish the interaction between the two proteins. Detailed insight in this matter might offer new clues on factors influencing structural stability of GBA and even find therapeutic application.

The relatively recent realization that defective GBA in GD patients and even carriers constitutes a major risk factor for developing alpha-synucleinopathies such as Parkinson's disease (PD) and Lewy-Body dementia has greatly raised the interest in the interplay between α-synuclein (α-Syn) and GBA[50,51]. The underlying cause for the remarkable association between GBA defects and PD risk is still enigmatic. There are conflicting reports on the relationship between the severity of the mutation in GBA and the risk for developing PD, although recently it has been proposed that in symptomatic GBA-deficient PD patients the severity of cognitive decline is influenced by the severity of the GBA mutation[52–54]. α-Syn abnormalities in GBA-deficient PD patients are not restricted to the brain. In such patients higher plasma oligomeric α-Syn levels were observed compared to controls as well as other PD patients[55]. Michelakakis and co-workers observed increased dimerization of α-Syn in the red blood cell membrane of GD patients[56,57].

Numerous reports on direct and indirect interaction of α-Syn and GBA are published in recent time. A clear picture has still not emerged. In the first place, some of the literature points to a direct inactivation of GBA by α-Syn[51,58]. For example, α-Syn was reported to interact with GBA via its C-terminal region in a pH-dependent way[51]. This complex did not form at neutral pH and N370S GBA presented decreased affinity for α-Syn[51]. In a more recent study membrane-bound α-Syn was shown to interact with GBA and inhibit enzymatic activity in sub-micromolar range both via substrate accessibility and turnover[58]. It was proposed that α-Syn could influence GBA-membrane interaction and/or induce conformational change influencing stability[58,60]. Of interest, it was reported that SapC protects GBA against inhibition by α-Syn[61]. Since SapC and α-Syn both have acidic regions it was suggested that they have overlapping binding sites on GBA[61]. Yap *et al.* recently proposed that α-Syn binding displaces GBA from the membrane by which it perhaps hampers access to substrate and disturbs the active site[60]. On the other hand, GBA significantly changes membrane-bound α-Syn by shifting helical residues away from the bilayer. It was speculated that in this manner lysosomal α-Syn breakdown is regulated[60]. Again, it still needs to be more precisely established how α-Syn binds to GBA and whether this interaction affects the structural stability of the enzyme.
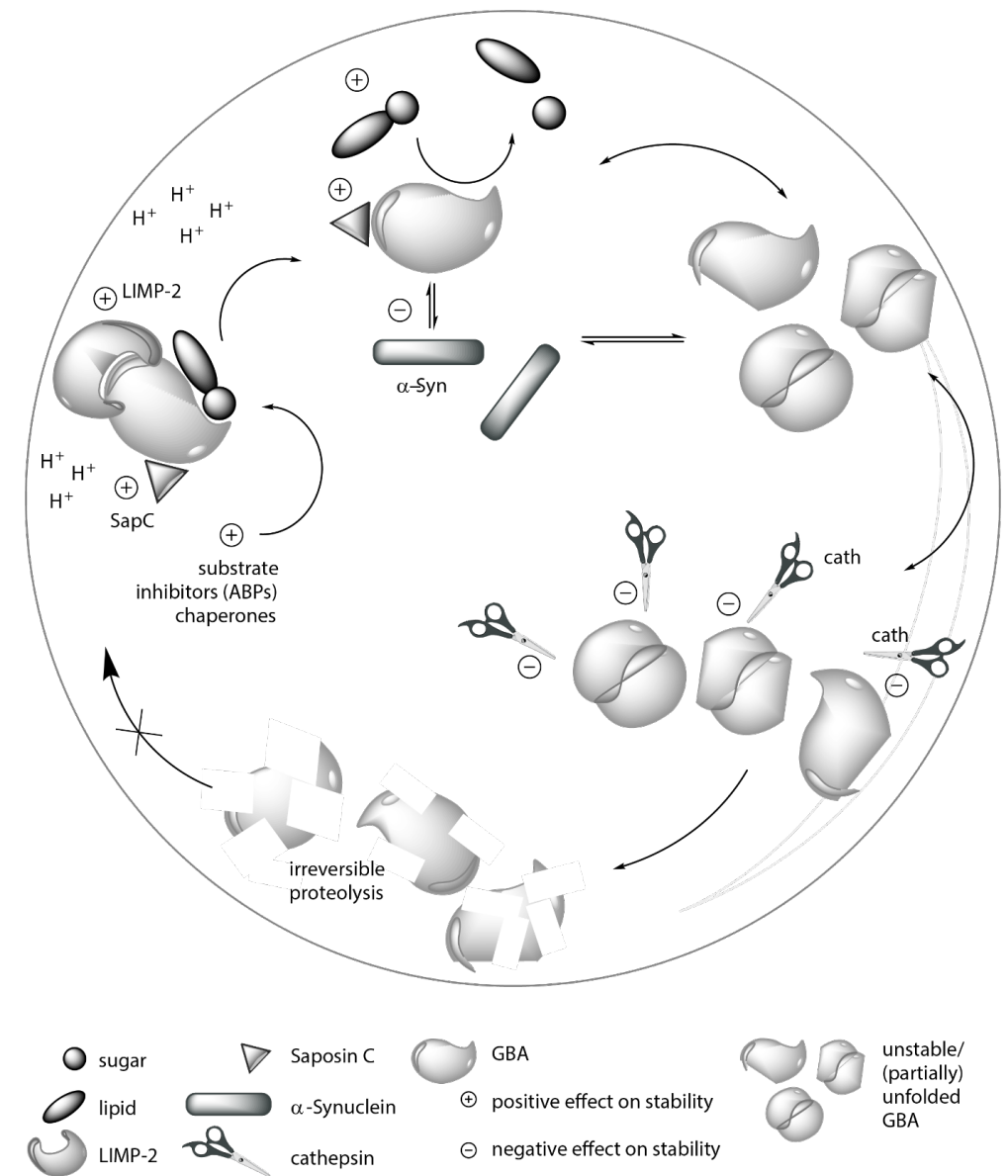
Figure 2. Positive and negative influences on GBA during its (lysosomal) life cycle.

In addition to a direct detrimental binding of GBA and α-Syn, functional deficiency of GBA has been claimed to cause lysosomal α-Syn accumulation and aggregation[50]. GlcCer was demonstrated to directly affect amyloid formation by stabilization of soluble α-Syn oligomeric intermediates[50]. Furthermore, elevated α-Syn was shown by Mazulli *et al.* to impair the intracellular transport of newly formed GBA to lysosomes and thus reduce the capacity of

lysosomes to degrade GlcCer[50]. Such mechanism would create a forward loop, resulting in ongoing α-Syn accumulation.

Importantly, recent studies indicate that augmentation of lysosomal GBA in the CNS of mice by adeno-associated virus-mediated expression of enzyme has positive effects on α-Syn accumulation and aggregation[62,63]. Of interest in this connection is also the recent demonstration that LIMP-2 may ameliorate α-Syn accumulation[64]. Deficiency of LIMP-2 in mice triggers accumulation of soluble as well as insoluble oligomeric α-Syn species in brain, probably as the result of lysosomal GBA deficiency and buildup of GlcCer or related metabolite[64]. The cell permeable LIMP-2 helix 5-derived peptide that promotes GBA capacity in lysosomes was found to diminish α-Syn in cultured cells[29].

Translation of fundamental knowledge to therapy.

As summarized in Table 1, multiple approaches to increase GBA are presently used or envisioned as therapeutic intervention for GD patients and individuals with GBA deficiency developing Parkinson's disease.

The seminal studies by Brady and co-workers identified supplementation of lysosomes of tissue macrophages with GBA as therapy approach for non-neuronopathic type 1 GD[65–67]. Indeed, ERT with macrophage-targeted, mannose-terminal recombinant GBA is proven efficacious for type 1 GD patients. Chronic bi-weekly enzyme infusion corrects and prevents organomegaly and hematological abnormalities in these patients[5]. New developments regarding ERT concern the use of different, more economic and saver production platforms for recombinant enzyme, for example plants[68]. Attention is also focused by pharmaceutical industry to additives increasing stability of therapeutic recombinant enzyme, to generation of fusion proteins able to cross the blood-brain barrier and to production of alternatively targeted recombinant GBAs to be used in combination with the present macrophage-targeted recombinant enzyme preparations.

An effective alternative treatment for type 1 GD is offered by substrate reduction therapy (SRT)[69–71]. This treatment modality makes use of oral administration of inhibitors of glucosylceramide synthase (GCS) and thus attempts to lower the intralysosomal flux of GlcCer. The first agent applied, and registered for 15 years, was N-butyl-deoxynojirimycin (*Miglustat; Zavesca*, Actelion Corp.)[69]. *Miglustat* is no potent and specific inhibitor of GCS, but nevertheless it was found to stabilize disease in mild type 1 GD patients[69]. More recently, *Eliglustat* (N-[(1R,2R)-1-(2,3-dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-pyrrolidin-1-ylpropan-2-yl]octanamide; *Cerdelga,* Genzyme Corp.) is also registered as SRT drug for type 1 GD. Clinical and biochemical responses to *Eliglustat* are on a par with high dose ERT[72–74]. Unfortunately, *Eliglustat* is not brain permeable and offers no prevention against neuropathology in severely affected type 2 and 3 GD patients. Moreover, *Eliglustat* is subject to CYP 2D6 metabolism, complicating its application in some patients due to aberrant metabolism of the drug. Considerable room remains for improvement of GCS inhibitors. More potent and brain permeable inhibitors are presently under investigation[75,76].

Lately much of the attention regarding therapeutic agents goes out to small molecule chaperones of GBA which can penetrate the blood-brain barrier and complement ERT. These should assist folding and improve transport of mutant GBAs to lysosomes. A very large number of structures has been proposed as candidate chaperones for GBA, for recent comprehensive reviews on the topic see references[77,78]. A major fraction of the studied chaperones are active-site specific iminosugars, either unmodified such as isofagomine (IFG), or *N*-alkylated such as NN-DNJ and NB-DNJ[77,78]. *N*-(*n*-nonyl)deoxynojirimycin (NN-DNJ) was the first molecule proposed as chaperone for GBA, in particular N370S GBA[79]. *Miglustat* (NB-DNJ) was next proposed to increase lysosomal activity of N370S GBA[80]. The potent competitive inhibitor IFG was also shown to augment mutant GBA activity[81,82]. However, a clinical trial did not substantiate a beneficial action of isofagomine in GD patients and further drug development was aborted by the pharmaceutical company involved (Amicus Corp.)[83]. Other classes are *C*-alkylated iminosugars comprising 1-azasugars, calystegines and DIX, amphiphilic bicyclic sp2-iminosugars comprising sp2-iminosugars related to the natural reducing alkaloid nojirimycin (NJ), such as 5N,6O-(N0-octyliminomethylidene) nojirimycin (39, NOI-NJ) or its 6-thio (40,6S-NOI-NJ) and 6-amino-6-deoxy (41, 6N-NOI-NJ) analogues, aminocyclitols such as *N*-octyl-β-valienamine (NOV) and aminosugars such as derivatives with the cis-1,2-fused (gluco)pyranose-2-alkylsulfanyl-1,3-oxazoline (PSO) structure[78]. Some of the chaperones presently pursued deserve special mentioning. Compelling evidence has been reported for the use of α-1-C-substituted imino-D-xylitols (DIXs) as GBA chaperones with low cytotoxicity[84]. APP (3-amino-3-hydroxymethylpyrano[3,2-b]pyrrol-2(1H)-one) compounds with differing aglycone moieties offer another potentially useful class of chaperones since they competitively inhibit GBA at neutral pH but this inhibition strongly decreases at acid (lysosomal) pH[85]. A conformationally locked C-glycoside based on the APP scaffold with an incorporated palmitoylamido segment as aglycone moiety was reported to boost N370S/N370S GBA activity with an efficacy comparable to that of Ambroxol (see below)[85].

Lately high throughput screening of large numbers of compounds has been used in the search for new GBA chaperones[86–90]. The traditional screens usually identify GBA chaperones which are concomitant inhibitors of enzyme activity. This impedes clinical application since there is only a small window between desired chaperone function and enzyme inhibition. Therefore, Patnaik *et al.* developed a new high throughput screen with which they identified a series of allosteric compounds that do not or hardly inhibit GBA but can still assist transport to lysosomes[91–94]. Specific pyrazolopyrimidines and salicylic acid derivatives ML198 and ML266 were reported to activate GBA and function as a chaperone and lack inhibitory activity[95]. Another class of selective non-iminosugar chaperones comprises quinazoline derivatives[96]. A new set of selective quinazoline derivatives displaying linear mixed inhibition was shown to stabilize GBA and increase intralysosomal N370S enzyme[97]. Moreover, a novel non-inhibitory small molecule chaperone NCGC607 was recently reported to augment GBA and decrease GlcCer levels in GD macrophages[98]. Additionally, GD iPSC-derived dopaminergic neurons were shown to have higher GBA activity, improved lysosomal translocation of GBA, diminished GlcCer levels and reversed build-up of α-Syn upon NCGC607 treatment[98]. Allosteric

activators of GBA are presently developed: one such compound LTI-291 (Lysosomal Therapeutics Inc; Allergan Corp.) is to be tested in PD with GBA abnormalities[99].

Some existing approved drugs for other disease conditions might also boost GBA. A thermal denaturation assay of 1040 FDA-approved drugs identified Ambroxol (ABX) as GBA interactor[100]. ABX is used for many years to treat airway mucus hypersecretion and hyaline membrane disease in newborns[101–103]. ABX turned out to be a pH-dependent, mixed-type inhibitor of GBA, with maximum inhibition at neutral pH[100,104,105]. The treatment of GD fibroblasts with ABX, including cells homozygous for L444P GBA, significantly augmented GBA and ABX-treated GD lymphoblasts displayed reduced GlcCer accumulation[100,104,105]. Macrophages derived from GD iPS cells showed phenotypic correction upon treatment with ABX, displaying diminished elevation of TNF-α, IL-1β and IL-6[106]. Additionally, ABX was found to reduce oxidative stress in fibroblasts of normal subjects, GD patients and GD carriers with and without PD[107]. Wildtype mice treated for one week with ABX showed increased GBA activity in spleen, heart and cerebellum[108]. Furthermore, wildtype as well as transgenic heterozygous L444P mice and mice overexpressing human α-Syn had significantly increased GBA activity and reduced α-Syn in the brain[109]. A pilot study reported improvement of GD specific parameters in ERT naïve GD patients receiving ABX[110]. Furthermore, ABX treatment of neuronopathic GD patients led to significantly augmented lymphocyte GBA activity and reduced GlcSph levels in cerebrospinal fluid[111]. Neurological manifestations such as myoclonus, seizures and pupillary light reflex dysfunction were stable during the treatment[111]. In a type 3 GD patient ABX treatment ameliorated epileptic attacks[112]. The findings made with ABX treatment of GD patients have led Ishay *et al.* to recommend simultaneous administration of ERT and ABX to carriers of mutant GBA at risk for PD[113].

To develop small compound stabilizers of GBA a distinct approach from the high throughput screening of compound libraries (and subsequent medicinal chemistry) described above might be considered, i.e. is to obtain rational leads from fundamental investigations on GBA. It has been recently recognized that GBA is catalytically more diverse as hitherto assumed (chapter 3, 4). Furthermore, there is increasing evidence that occupancy of the catalytic pocket of GBA favors structural stability of the enzyme (chapter 2). One of the newly identified interactors with GBA is cholesterol. The sterol is able to reach the catalytic pocket as indicated by competition of pacGlcCer labeling of GBA (chapter 3) and it acts as acceptor in transglucosylation (chapter4). Moreover, GlcChol also serves as substrate for GBA (chapter 4). A careful examination of potential chaperone or inhibitory actions of (glycosylated) cholesterol or mimics thereof might be of interest. Particularly intriguing is also the affinity of GBA for β-xylosides that are poorer substrates than corresponding β-glucosides. Again, it seems worthwhile to investigate the impact of β-xylosides on GBA in living cells and organisms.

The knowledge on proteins interacting with GBA in lysosomes might also be translated to (additive) therapy approaches. For example, it is now apparent that LIMP-2 is critical for lysosomal GBA activity and α-Syn clearance[64]. Indeed, it was earlier shown that defects in the SCARB2 gene also constitute an increased risk for developing PD[114]. A cell-penetrating LIMP-2

helix 5-derived peptide has been shown to increase lysosomal GBA and decrease α-Syn levels[29]. Further investigations on therapeutic use of such peptides are warranted. The same holds for potential chaperoning SapC peptides. Recently, SapC was chemically synthesized and found to activate and stabilize GBA and moreover guard it from cathepsin D breakdown[115–117]. Cathepsin D was chosen because the authors previously found positive effects of its inhibitor on GBA activity[47] (in contrast to our results, data not shown). A major challenge with peptide supportive therapy is the limited ability of peptides to cross the blood-brain barrier. Design of cell penetrating peptides able to cross the BBB will be an essential next step for developing a realistic therapeutic agent.

A completely novel therapeutic approach employs pharmacological induction of the heat shock protein, HSP70[118–120]. HSP70 has been shown to protect lysosomes during stress and stabilize acid sphingomyelinase (ASM), an enzyme deficient in Niemann-Pick types A and B[118]. Niemann-Pick type B fibroblasts were found to display corrected lysosomal morphology upon treatment with recombinant HSP70[118]. This finding was extended to fibroblasts of eight different lysosomal storage disorders[120]. It has recently become clear that Arimoclomol potently induces endogenous HSP70[120]. Arimoclomol is presently tested in phase II studies by Orphazyme Corp. as experimental drug to treat Amyotrophic Lateral Sclerosis (ALS). A clinical study for the lysosomal storage disorder NPC is also approved by the FDA. Investigation of the outcome of Arimoclomol-mediated induction of HSP70 in neuronopathic GD will be of great interest.

A true cure for GD would in principle be offered by gene therapy. In the case of type 1 GD, successful bone marrow transplantation has been reported to result in major clinical correction[121–124], indicating that correction of GBA content in blood cells is sufficient for a satisfactory clinical response. The risk associated with allogenic bone marrow transplantation has limited the application. The combination of genetic correction of own hematopoietic cells of GD patients is appealing[125]. Recently very promising results have been obtained in a type GD mouse model with viral vectors allowing macrophage specific expression of GBA[124]. The recent advances with CRISPR/Cas technology should in the future offer possibilities for substituting aberrant genetic information by the correct one in the endogenous GBA alleles of GD patients[126,127].

Table 1. Existing and proposed (theoretical) therapy options for Gaucher disease.

Therapy	Agent	Mode of action
Existing therapies		
ERT	Recombinant GBA1 (Imiglucerase/ Cerezyme)	Enzyme supplementation
	Recombinant GBA1 Taliglucerase alfa (Elelyso)	Plant produced Enzyme supplementation
SRT	GCS inhibitor (Cerdelga/ Eliglustat)	Substrate reduction
Iminosugar chaperone	NB-DNJ (Zavesca)	Chaperone, transport promotion & Substrate reduction
Experimental therapies		
Iminosugar chaperone	NN-DNJ	Chaperone, Folding promotion
	Isofagomine (Plicera)	Chaperone, transport promotion
Chemical chaperones; Small compounds	Ambroxol	Chaperone, transport promotion
	APPs	Chaperone
	ML155, ML156	Chaperone
	ML198, ML266	Chaperone
	Quinazoline derivatives	Chaperone, transport promotion
	NCGC607	Chaperone
Allosteric compound	LTI-291	Activator, transport promotion
Heat Shock Protein response	Arimoclomol	Enzyme stabilization, refolding
Allosteric protein chaperones	LIMP-2 peptide	Transporting chaperone
	sapC (peptide)	Lysosomal chaperone
Gene therapy	DNA construct	cDNA/gene correction
Theoretical therapies		
Chemical chaperones; Small compounds	Cholesterol based compounds	Active site chaperone
	Xylosyl-β-glucsosides	Active site chaperone
Protease inhibition	Cathepsin inhibitor	Lysosomal proteolysis protection

The adaptations to GBA deficiency.

A final topic for discussion forms the relative recent realization that deficiency of GBA leads to adaptations with potential pathological consequences (see also chapter 5 for a review). Primary GBA deficiency occurs in GD patients due to defects in their two GBA alleles. A secondary deficiency of GBA occurs in AMRF (action myoclonus-renal failure syndrome) patients due to defects in the SCARB2 gene encoding LIMP-2[128,129]. Reduced GBA is also noted in patients with Niemann-Pick type C (NPC) disease due to defects in NPC1 or NPC2, proteins mediating the export of cholesterol from lysosomes[130].

One adaptation to lack of GBA in GD patients is the increased formation of gangliosides, likely due to permanent excess of the precursor GlcCer[131–133]. Elevated gangliosides like GM3 seem to promote the noted insulin resistance in GD patients[132]. The same metabolic abnormality occurs frequently in obese individuals in which formation of ceramide and subsequent glycosphingolipids is chronically increased[133,134]. The insulin receptor is present at the cell's plasma membrane in glycosphingolipid-rich rafts and a disturbed environment of gangliosides is thought to cause impaired insulin signaling. This mechanism is substantiated by findings in cell and animal models[135,136]. For example, mice deficient in ganglioside synthesis are protected against diet-induced obesity and the pharmacological reduction of GlcCer synthesis has been shown to increase insulin signaling in adipocytes and in various mouse and rat models of obesity-induced insulin resistance[134]. It has been debated whether excessive glycosphingolipids or elevated ceramide underlies impaired insulin signaling during obesity. A consensus is now reached in which excessive gangliosides are thought to cause insulin insensitivity in adipocyte, whereas excessive ceramide is the primary toxic agent in muscle[137,138]. Likely, the common chronic tissue inflammation during obesity drives the pathology onwards by promoting excessive ceramide synthesis and subsequent formation of glycosphingolipids that on their turn again promote inflammation.

A further adaptation to deficient GBA concerns the cytosol-faced membrane β-glucosidase GBA2[139–141]. The 3D-structure of GBA2 has not been resolved yet, but it is known that the enzyme's catalytic pocket is inserted in the cytosolic membrane leaflet. GBA2 is found to be increased during GBA1 deficiency[141,142], likely a compensation to regulate cellular GlcCer[130]. In mice with a conditional knockout of GBA in white blood cells concomitant absence of GBA2 reduces hepatosplenomegaly, cytopenia and skeletal disease[143]. Likewise, genetic loss of GBA2 as well as pharmacological inhibition of the enzyme is found to ameliorate manifestations of NPC in mice, particularly the loss of motor neurons[130]. These findings point to detrimental consequences of GBA2 over-activity during deficiency of GBA. On the other hand, several patients with defects in the GBA2 gene developing spastic paraplegia and cerebellar ataxia have recently been identified[144–149]. This suggests that a marked reduction of GBA2 activity, at least in some individuals, has negative consequences. Puzzling in this connection is that GBA2 deficiency in mice does not cause any overt pathology except for partially blocked spermatogenesis and that the treatment of GD and NPC patients with *Miglustat,* a nanomolar inhibitor of GBA2, is generally well tolerated[71,150,151]. In view of

this conundrum of conciliating the various findings a closer look to GBA2 is of interest. It has recently been demonstrated that the enzyme acts as a transglucosylase, reversibly transferring glucose from GlcCer to cholesterol to yield ceramide and GlcChol[22]. It can presently not be excluded that other metabolites also participate in these transglucosylation reactions. In view of this, changes in ceramide and glucosylated metabolites might be implicated in the noted beneficial effects of inhibition of GBA2 as well as in the neuropathology shown by GBA2-deficient individuals.

Another key adaptation to GBA deficiency is the active formation of glucosylsphingosine (GlcSph) by de-acylation of GlcCer through lysosomal acid ceramidase[152]. Most cell types of GD and AMRF patients manage to limit GlcCer accumulation through this pathway[153]. Formed GlcSph may leave cells and even the body via bile and urine[152]. As a consequence, plasma GlcSph is several hundred-fold increased in symptomatic GD patients[154]. This abnormality is however not without health risk. It was observed that excessive plasma GlcSph is linked to B-cell lymphoma[155]. Nair *et al*. recently reported that GlcSph in GD patients acts as auto-antigen that drives B-cell proliferation and increase the risk for associated multiple myeloma, a leukemia with increased incidence in GD patients[156,157]. A similar pathway of acid ceramidase-mediated formation of sphingoid bases from accumulating glycosphingolipid is active in other inherited glycosphingolipidoses[158]. For example, in α-galactosidase A deficient Fabry disease patients the water-soluble base lysoGb3 (globotriaosylsphingosine) is generated from accumulating Gb3 (globotriosylceramide)[159]. Plasma lysoGb3 is markedly elevated and lysoGb3 is present in urine[160]. Excessive lysoGb3 has been demonstrated to be toxic for nociceptive peripheral neurons and podocytes, contributing to the common pain in the extremities and the renal failure of Fabry disease patients[161,162]. Another example forms the neurotoxic galactosylsphingosine in Krabbe disease, the sphingoid base formed from the primary storage lipid galactosylceramide in the galactocerebrosidase-deficient patients[163]. The quantitative detection of glycosphingoid bases in plasma samples by UPLC-ESI-MS/MS using ^{13}C-encoded internal standards has enormously improved diagnosis of GD and Fabry disease patients as well as the monitoring of efficacy of therapeutic intervention[154,160,164] (addendum 2). Another important application for ^{13}C-encoded glycosphingolipids is laid in their use for detailed investigation of *in vivo* lipid metabolism and the impact of (potential) therapeutic agents[152].

Perspectives

GBA is a truly fascinating enzyme. Fifty years after its discovery by Brady and colleagues[165] and despite subsequent intense research, the enzyme today still poses multiple challenging questions. GBA differs from other lysosomal hydrolases in fundamental aspects. Newly formed GBA is uniquely transported to lysosomes, bound to the integral membrane protein LIMP-2, traversing distinct compartments from the other mannose-6-phosphate receptor bound acid hydrolases[26,166,167]. The evolutionary pressure for this unique sorting is still not elucidated. The (transient) interaction of GBA with LIMP-2 may have physiological implications. LIMP-2 (*aka* SCARB2) has been proposed to harbor a lipid transport function similar to the related proteins SCARB1 and SCARB3[168]. It is appealing to speculate that a product of GBA is channeled via LIMP-2 from lysosomes to the cytosol. The recent insight that GBA also interacts with cholesterol and can generate GlcChol might be relevant in this connection. It might be hypothesized that the GBA/LIMP-2 complex plays a role in (glucosylated) cholesterol transport from lysosomes. Closer investigation of the impact of cholesterol and its metabolites on GBA and GlcCer metabolism is highly warranted. In view of this, the presence of active GBA in the extracellular layer of skin rich in GlcCer and cholesterol is intriguing[24]. A local role for GBA in the fine-tuning of glucosylated sterols and sphingolipids with impact on skin permeability is a hypothetical possibility that should be further investigated.

This thesis research primarily focused on factors influencing stability and activity of GBA in lysosomes. The interest in this topic has enormously increased after GBA deficiency was implicated in neurodegenerative conditions like Parkinson's disease and Lewy-Body dementia. Increasing the lifespan of GBA molecules by inhibition of a specific cathepsin involved in the enzyme's lysosomal breakdown is appealing. However, the conducted research on this topic did not render a suitable target cathepsin. A more attractive approach to increase lifespan of GBA seems the stabilization of GBA conformation. Own research and literature suggests that there are multiple potential targets for this. Firstly, there are indications that both LIMP-2 and saposin C by binding to GBA inside lysosomes may stabilize the enzyme and increase its hydrolytic activity. The amphiphilic polypeptide α-synuclein seems to interfere with these beneficial interactions and therefore impairs GBA. It can be envisioned that in the near future polypeptides, like LIMP-2 fragments and potentially also saposin C fragments, may be selected to promote stability and activity of GBA inside lysosomes. The induction of specific proteins, like HSP70, able to actively refold proteins inside lysosomes is also offering an exciting new option for augmenting GBA and deserves further investigation. Secondly, it has become clear that small compounds may influence the folding and structural stability GBA in cells. Chemical chaperones directly interacting with the catalytic pocket of GBA receive considerable attention, in particular glyco-mimetics interacting with the glycon binding site of the enzyme. An intrinsic disadvantage of these type of agents is their ability to effectively inhibit enzyme activity when present at too high dose. This offers considerable challenges in their practical use in patients where ideally concomitantly in various tissues and cell types the desired concentrations is reached. A new promising development is the identification of small compounds that allosterically (beyond the catalytic pocket) boost GBA. Stemming from own research the impact of cholesterol and β-xylosides on GBA also deserves further investigation in this light. Finally, the more direct approaches to increase lysosomal GBA such as enzyme replacement therapy and in particular gene therapy remain to hold great therapeutic potential for Gaucher disease as well as Parkinson's disease induced by GBA deficiency.

The recent discovery of unexpected catalytic actions of GBA opens up new exciting research fields. It will be interesting to identify which glucosylated metabolites beyond GlcCer and GlcChol naturally occur in the human body and what the precise role of GBA is in their

metabolism. In this connection, the potential natural occurrence of β-xylosylated metabolites should also be carefully examined. In addition, the possible impact on GBA of food-derived exogenous hydrophobic β-glucosides and β-xylosides as well as sterol-like structures should be taken in mind.

In conclusion, half a century after its discovery GBA still resembles the mythological Sphinx, a multi-faceted creature posing riddles.

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