

Cover Page



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

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Summary

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This thesis describes biochemical investigations of glucocerebrosidase (GBA), the lysosomal β -glucosidase that is deficient in Gaucher disease (GD). Central in the performed research was the examination of factors influencing the intralysosomal stability and half-life of GBA. The investigations made use of new chemical biology tools such as activity based probes (ABPs) and photo-activatable and clickable (PAC) lipids.

Chapter 1 concerns the lysosomal cathepsins involved in proteolytic breakdown of GBA. More detailed knowledge on the responsible enzymes might open a novel avenue for therapy through boosting lysosomal GBA levels by inhibition of a specific protease. Earlier work had revealed that GBA is degraded in lysosomes by cysteine cathepsins, as indicated by the protective effect of leupeptin, a broad-specific protease inhibitor.

The conducted investigation showed that also E64d and the derived activity-based probe DCG-04, protect lysosomal GBA in normal and GD patient fibroblasts and lymphoblasts against proteolytic degradation, as revealed by enzymatic activity assays and labelling of active GBA with activity-based probes. Exposure of cultured GD lymphoblasts to E64d led to a reduction of glucosylsphingosine, the base formed from accumulating GlcCer, indicating a functional correction in GBA capacity. The candidate proteases involved in lysosomal GBA breakdown were narrowed down to cathepsins B, F and L. However, individual reduction of these cathepsins by shRNA or CRISPR-Cas technology was insufficient to significantly reduce the turnover of GBA in cultured cells. Apparently, multiple cysteine cathepsins are able to degrade GBA in lysosomes and need to be concomitantly inhibited to augment lysosomal GBA. Such approach is considered unattractive as therapy given the expected side effects.

Chapter 2 describes the effect of the occupancy of the catalytic pocket of GBA regarding its structural stability *in vitro* and *in vivo*. Glycomimetics are presently designed as chemical chaperones to promote folding and stabilization of GBA in GD patients, but evidence for *in vivo* efficacy of this approach is scarce.

The performed study made use of amphiphilic cyclophellitol-derived activity-based probes (ABPs) that irreversibly bind to the catalytic nucleophile E340. The potent reversible inhibitor isofagomine, a former therapeutic candidate chaperone for GBA, was used for comparison. Cyclophellitol ABPs increased very markedly stability of recombinant GBA *in vitro* as shown by thermodynamic measurements and relative resistance to tryptic digestion. The stabilizing effect of isofagomine on structural stability of pure GBA was less potent. Stabilization by the cell permeable ABP of GBA in cultured normal and Gaucher disease patient cells was reflected by the clear increase in enzyme. GBA in liver of wild-type mice infused with ABP was also found to be stabilized. In conclusion, occupancy of the pocket of GBA by amphiphilic ABPs markedly stabilizes the enzyme. This finding supports the concept of reversible chaperones of GBA as therapeutic agents for Gaucher disease.

Chapter 3 addresses the investigation of GBA with chemical biology tools: pacGlcCer and fluorescent β -glucose configured cyclophellitols binding to the catalytic nucleophile residue E340 in mechanism-based manner. Structure-function relationships in GBA have so far been studied with conventional enzymology and crystallography and relatively little knowledge exists on the aglycon binding moiety of GBA.

The presented study describes that the photo-activatable (diazirine functionalized) and clickable (alkyne containing) pacGlcCer binds with high affinity to the catalytic pocket of active GBA, following the pH optimum of enzymatic activity. GBA hydrolyzes pacGlcCer, further substantiating its ability to enter the catalytic pocket. The binding of pacGlcCer to GBA is completely competed by the reversible inhibitor AMP-DNM as well as the irreversible inhibitors conduritol β -epoxide, cyclophellitol and derived ABP with a fluorophore tag. The labeling of GBA by pacGlcCer is also competed by the substrates glucosylsphingosine, GlcCer and 4-methylumbelliferyl- β -glucose. Cholesterol, an acceptor in the transglucosylation reaction of GBA, also potently inhibits the binding of pacGlcCer, but not that of ABP. The findings indicate specific labeling of GBA by pacGlcCer via its catalytic pocket. It is envisioned that pacGlcCer and ABP labeling of GBA can be used in screens identifying interacting small compounds. To conclude, the available tools offer novel possibilities to probe the glycon- and aglycon-binding sites of the catalytic pocket of GBA.

Chapter 4 reports on the interaction of the lysosomal membrane protein LIMP-2 with GBA. GD is caused by mutations in GBA itself, whereas Action Myoclonus Renal Failure (AMRF) is due to mutations in LIMP-2 that governs transport of newly formed GBA to lysosomes. The deficiency in LIMP-2 results in faulty secretion of GBA in most cell types. Intriguingly, AMRF patients do not develop the characteristic glucosylceramide (GlcCer)-laden macrophages (Gaucher cell) that are a hallmark of GD. Macrophage-targeted GBA with terminal mannose residues in its N-glycans is successfully used in enzyme replacement therapy (ERT) of GD, but for AMRF no treatment is presently available.

The conducted investigation examined the supplementation of LIMP-2 deficient cells with therapeutic mannose-terminated GBA. Studies were conducted with normal and AMRF fibroblasts as well as normal LIMP-2 deficient HEK293 cells transduced with mannose receptor. In LIMP-2 deficient cells, the endocytosed enzyme was found to be abnormally fast degraded in lysosomes. This suggests that transient interactions of GBA with LIMP-2 in the lysosome stabilize the conformation of the enzyme and slow down its proteolytic breakdown. It furthermore implies that the LIMP-2 in lysosomes determines the efficacy of supplementing the organelles with GBA by ERT. In conclusion, the study revealed that LIMP-2 fulfils a dual purpose in the life cycle of GBA, as transporter and as intralysosomal chaperone.

Chapter 5 concerns the fundamentally different clinical manifestation of GD and AMRF, two inherited diseases in which GBA is reduced. In GD, GBA is mutated, whereas in AMRF the normal enzyme is faulty secreted instead of transported to lysosomes. The cellular and biochemical basis for the different symptomatology of GD and AMRF was examined employing a LIMP-2 deficient mouse model.

The study first addressed the possibility that LIMP-2 deficiency not only affects GBA but also other lysosomal proteins. No abnormalities in lysosomal matrix proteins, except GBA, were observed for lysosomes isolated from liver of LIMP-2-deficient mice. Next, LIMP-2 deficient mice were examined regarding residual GBA and lipid abnormalities in various tissues. A variable deficiency of GBA along tissues of LIMP-2 deficient mice was observed, both by enzymatic activity assays and labelling of active enzyme with an activity-based probe. A high residual GBA was detected in leukocytes of LIMP-2 deficient mice, explaining the absence of macrophage-related symptoms in AMRF patients. Evidence was obtained for endocytotic re-uptake of GBA by white blood cells contrary to fibroblasts. Lipid analysis showed that GlcCer is hardly increased in tissues of LIMP-2 deficient mice and that its levels poorly correlate with residual GBA. In contrast, glucosylsphingosine and glucosylated cholesterol are increased and their levels in tissues negatively correlate with GBA levels. Glucosylsphingosine and glucosylated cholesterol were also found to be increased in isolated lysosomes of LIMP2-deficient liver. To conclude, LIMP-2 deficiency results in cell-type specific reduction of GBA and adaptive changes in glycosphingolipid metabolism.

Chapter 6 reviews the evidence for metabolic adaptations in two glycosphingolipidoses, GD and Fabry disease (FD) caused by defects in GBA degrading GlcCer and alpha-galactosidase A (GLA) degrading globotriaosylceramide (Gb3), respectively. The accumulation of the primary storage lipids in cells and tissues of GD and FD patients tends to level with age, suggesting the induction of metabolic adaptations. An important adaptation in GD as well as FD is the active de-acylation of accumulating GSLs in lysosomes by the action of the enzyme acid ceramidase. Thus, the lysosomal lipid storage is limited through formation of glucosylsphingosine (GlcSph) in GD and globotriaosylsphingosine (lysoGb3) in FD. Excessive GlcSph and lysoGb3 are considered to be toxic and contribute to specific symptoms such as multiple myeloma in GD patients and neuropathic pain and renal failure in FD patients. Another adaptation in metabolism in GD takes place beyond the lysosome, involving the enzyme GBA2 located in the cytoplasmic leaflet of membranes of the ER and Golgi apparatus. GBA2 allows extra-lysosomal degradation of GlcCer and concomitantly generates ceramide (Cer) and glucosylated cholesterol. Specific targeting of the toxicity caused by metabolic adaptations in GD and FD as therapy approach is discussed.

Chapter 7 deals with the catalytic versatility of GBA, a β -glucosidase that until recently was considered to solely cleave GlcCer to Cer and glucose in lysosomes. Special attention is paid to transglycosylation catalyzed by GBA and its specificity for the glycon moiety of substrate.

GBA is known to hydrolyze β -glucosidic substrates and more recently to also transglucosylate cholesterol to cholesterol- β -glucoside (GlcChol). The described study focused on the ability of GBA to metabolize β -xylosides. It is firstly shown that GBA also cleaves 4-methylumbelliferyl- β -D-xylose (4MU- β -Xyl) *in vitro*, being stimulated in this activity by the activator protein saposin C. Next, GBA is shown to *in vitro* transxylosylate cholesterol using 4MU- β -Xyl as sugar donor. Formed xylosyl-cholesterol (XylChol) acts as subsequent acceptor

to render di-xylosyl-cholesterol. Sequential exposure of GBA and cholesterol to 4MU- β -Xyl and 4MU- β -Glc results in formation of GlcXylChol. Similar to GBA, the cytosolic β -glucosidase GBA3 shows *in vitro* β -xylosidase and transxylosylase activity, in contrast to the membrane-bound GBA2 that lacks activity towards β -xylosides. Cultured cells also generate xylosylated cholesterols when exposed to 4MU- β -Xyl in their medium. This synthetic reaction is enhanced by the drug U18666A causing an increase in lysosomal cholesterol. Prior inactivation of GBA in cells with conduritol B-epoxide prohibits formation of xylosylated cholesterols. In conclusion, further catalytic versatility of GBA is revealed: the enzyme may act as β -glucosidase, β -xylosidase, transglucosylase and transxylosylase. The physiological relevance of the noted ability of GBA to metabolize β -xylosides is presently unclear and warrants further examination.

The **Discussion** reviews the present insights into GBA in health and disease. In this connection, the molecular basis and clinical manifestation of Gaucher disease and Action Myoclonus Renal Failure syndrome are discussed, including the metabolic adaptations to GBA deficiency.

Particular attention is paid to the lysosomal structural stability of GBA and associated resistance against proteolytic degradation by cysteine cathepsins. Literature findings and novel own results on this topic are discussed. New technology to study GBA by labeling with GlcCer and cyclophellitol derived probes is introduced and the application is described. Unresolved research questions on GBA and related disease conditions are identified. As future research objective the translation of fundamental knowledge on GBA to effective therapy of neuropathic Gaucher disease and other disease conditions caused by enzyme reduction are discussed.

The **Addendum** describes a newly developed multiplex assay for simultaneous quantitation of (lyso)-glycosphingolipid bases in plasma samples by use of UPLC-ESI-MS/MS with identical ^{13}C -encoded internal standards. Glycosphingolipids in the same plasma sample can also be quantified following microwave-assisted de-acylation to glycosphingoid bases.