



Universiteit
Leiden
The Netherlands

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

Oussoren, S.V.; Oussoren S.V.

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>

Version: Not Applicable (or Unknown)

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/55842>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/55842> holds various files of this Leiden University dissertation

Author: Oussoren, Saskia

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

Date: 2017-09-28

Augmentation of lysosomal
glucocerebrosidase by the
inhibition of its lysosomal
proteolysis

1

Augmentation of lysosomal glucocerebrosidase by the inhibition of its lysosomal proteolysis

Based on

S.V. Oussoren¹, S. Hoogendoorn², M. Verdoes³, S. Scheij⁴, M. Verhoek¹, M. Artola²,
R.G. Boot¹, H.S. Overkleeft², J.M.F.G. Aerts¹

¹Dept. of Medical Biochemistry, LIC, Leiden University

²Dept. of Bio-Organic Synthesis, LIC, Leiden University

³Dept. of Tumor Immunology, RIMLS, Nijmegen

⁴Dept. of Medical Biochemistry, AMC, University of Amsterdam

Manuscript in preparation

Abstract

Patients suffering from the lysosomal storage disorder Gaucher disease (GD) have defects in lysosomal glucocerebrosidase (GBA) and consequently an impaired turnover of the glycosphingolipid glucosylceramide (GlcCer). GBA is known to be degraded by lysosomal cysteine cathepsins: the broad-specific inhibitors leupeptin and E64d protect lysosomal GBA in GD patient fibroblasts and lymphoblasts as revealed by labelling of enzyme with activity-based probes and enzymatic activity assays. Inhibition of the proteolytic GBA turnover might in principle offer a novel therapeutic avenue to increase lysosomal GBA levels. Indeed, we observed that the inhibition of multiple cysteine cathepsins with E64d in cultured GD lymphoblasts results in a reduction of glucosylsphingosine, the base formed from accumulating GlcCer. Thus, a functional correction in GBA capacity can be accomplished in this manner. We next studied the contribution of various cysteine proteases in the lysosomal breakdown of GBA using activity-based probes with an E64 scaffold and shRNA or CRISPR-Cas mediated reduction of specific cysteine cathepsins. The candidate proteases predominantly involved in lysosomal GBA breakdown were first narrowed down to cathepsins B, F and L. Deletion of each of these cathepsins was insufficient to significantly reduce the turnover of GBA in cultured cells. We therefore conclude that multiple cysteine cathepsins are able to degrade GBA in lysosomes and would need to be concomitantly inhibited to render the desired increase in lysosomal GBA level. Such approach is unattractive since it would lead to a broad interference in lysosomal turnover of proteins.

1

Introduction

Patients with Gaucher disease (GD) are deficient in the activity of glucocerebrosidase (GBA), a lysosomal β -glucosidase encoded by the *Gba* gene at locus 1q21¹⁻³. Several hundred mutations have been identified in *Gba* of GD patients of which many cause single amino acid substitutions in the enzyme⁴. GBA is a 495-amino acid polypeptide with four N-linked glycans at Asn19, Asn59, Asn146 and Asn270¹. Inside lysosomes, GBA degrades glucosylceramide (GlcCer), a crucial step in cellular glycosphingolipid turnover⁵. The degradation of GlcCer by GBA is assisted by saposin C, as indicated by the consequences of inherited defects in this activator protein⁶. Recently it has become clear that naturally occurring glucosylated cholesterol is also a physiological substrate for the enzyme⁷. All cells of GD patients have impaired GBA, but macrophages in patient tissues most prominently accumulate GlcCer⁸⁻¹⁰. The lipid-laden macrophages of GD patients (Gaucher cells) produce and secrete specific proteins^{10,11}. Moreover, by the action of acid ceramidase GBA-deficient cells generate from accumulating GlcCer through de-acylation the water-soluble base glucosylsphingosine (GlcSph)¹¹. Elevated plasma levels of GlcSph and Gaucher cell-derived chitotriosidase, CCL18, gpNMB and sCD136 are employed as biomarkers to monitor disease progression¹²⁻¹⁷. The clinical presentation of GD is remarkably heterogeneous, ranging from fatal symptoms in skin and brain early in life to a virtually asymptomatic course of disease¹. The most common manifestation of GD among Caucasians does not involve the central nervous system (non-neuronopathic type 1 GD). Marked hepatosplenomegaly, thrombocytopenia and skeletal disease are characteristic symptoms of type 1 GD patients¹. Hetero- and homo-allelic presence of mutant *Gba* coding for N370S GBA, the prevalent mutation among Caucasian GD patients, is associated with a non-neuronopathic type 1 course of disease^{1,18,19}.

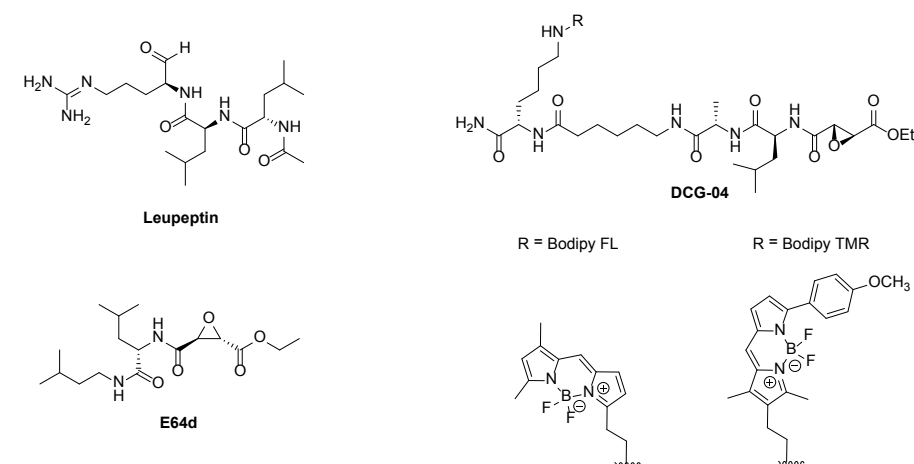
Otherwise, *Gba* genotype – GD phenotype correlations are relatively poor, explicitly illustrated by the occurrence of phenotypically discordant monozygotic twins^{20,21}. N370S GBA is almost normally folded in the endoplasmic reticulum and transported to lysosomes, but the mutant enzyme presents catalytic abnormalities and reduced stability^{22,23}. Many of the other mutations in GBA observed in GD patients lead to defective folding and reduced transport to lysosomes²⁴. One example in this respect is L444P GBA²⁵. Only a small fraction of newly formed L444P enzyme is correctly folded and transported to lysosomes. Most L444P GBA molecules are destined for degradation in proteasomes. Homozygosity for L444P GBA is usually accompanied by a severe neuropathic disease, again with marked individual variability¹. The 3D structure of the enzyme has been solved by X-ray diffraction crystal analysis, revealing a typical (β/α)₈ TIM barrel catalytic core domain III, a three-strand antiparallel β -sheet flanked by a loop and a perpendicular strand (domain I) and an Ig-like fold formed by two β -sheets (domain II)^{25,26}. *In silico* molecular modeling of the 3D structure does not render a reliable prediction of clinical severity of amino acid substitutions in GBA. Crystallography has confirmed earlier biochemical studies indicating that GBA is a retaining β -glucosidase, employing the double-displacement mechanism for catalysis in which Glu340 acts as nucleophile residue and Glu235 as acid/base residue^{27,28}. As reaction intermediate the glucose of the substrate GlcCer becomes covalently linked to E340 and is released by subsequent

attack of a hydroxide. Based on this is the irreversible inhibition of GBA by conduritol B-epoxide and cyclophellitol that form permanent conjugates with Glu340²⁹. Cyclophellitol scaffolds have been successfully used to design functionalized activity-based probes allowing in situ visualization of active GBA molecules^{30,31}.

The life cycle of GBA is fundamentally different from that of most other lysosomal hydrolases^{10,19}. In general, lysosomal hydrolases are glycoproteins, acquiring in the Golgi apparatus mannose-6-phosphate recognition signals in their N-glycans, allowing binding to mannose-6-phosphate receptors and subsequent delivery to lysosomes. In sharp contrast to this, the glycans of GBA are predominantly complex-type and lack mannose-6-phosphate groups³². Newly formed GBA is transported to lysosomes through binding to the triple helical structure in the apical region of integral membrane protein LIMP-2 (lysosome integral membrane protein 2, encoded by the *Scarb2* gene) with trafficking information in its cytoplasmic tail³³. The GBA-LIMP-2 complex reaches late endosomes/lysosomes where the enzyme dissociates at the local acid pH³⁴. Defects in LIMP-2 cause major reductions in GBA in most cell types except mononuclear phagocytes, likely contributing to the different clinical picture of GD and action myoclonus renal failure syndrome (AMRF), due to LIMP-2 deficiency³⁵. Trafficking of newly formed GBA from the ER to lysosomes takes relatively long and the half-life of GBA in lysosomes is only a few days, at least as observed in the cultured cells studied^{36,37}. GBA does not undergo proteolytic processing but is subject to modification of its N-glycans by the action of lysosomal glycosidases³⁸.

Brady and colleagues developed so-called enzyme replacement therapy (ERT), an approach based on two-weekly intravenous administration of macrophage-targeted GBA³⁹. ERT has proven to offer a very effective treatment for type 1 GD patients, resulting in impressive corrections in organomegaly and hematological abnormalities accompanied by corrections in biomarkers⁴⁰. Presently several recombinantly produced GBA preparations are registered for ERT of type 1 GD^{41,42}. Neurological manifestations in more severely affected GD patients are not prevented by ERT since the enzyme does not pass the blood-brain barrier⁴³. More recently, substrate reduction therapy (SRT) has been developed as alternative treatment for type 1 GD⁴⁴. Here, oral administration of an inhibitor of glucosylceramide synthase aims to reduce bodily GlcCer. Two inhibitors are registered for SRT of type 1 GD: *Miglustat* (N-butyldeoxynojirimycin) and lately the more potent *Eligustat* (*N*-[(1*R*,2*R*)-1-(2,3-Dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-(1-pyrrolidinyl)-2-propanyl]octanamide). Both drugs have been reported to result in clinical benefit to type 1 GD patients^{45,46}. Another approach actively pursued by academic researchers and pharmaceutical industry is based on small compound chaperones, assisting folding of mutant enzyme in the ER^{47,48}. Theoretically, some chaperones might also promote structural stability of GBA inside the lysosome and thus reduce sensitivity for proteolytic degradation. Small compound therapies for GD are appealing since they might provide treatment of neuropathology in GD, an unmet clinical need. An impairment of GBA, even at the level of GD carriers, has been found to significantly increase the risk for development of alpha-synucleinopathies⁴⁹.

Protease Inhibitors:



Scheme 1. Overview of the protease inhibitors used in this study. Top left: Leupeptin; reversible inhibitor of serine and cysteine proteases. Middle left: E64d; the cell-penetrable form of E64, irreversible inhibitor of cysteine proteases. Lower left: DCG-04; the activity based probe based on E64. Lower right: Bodipy FL and Bodipy TMR; fluorescent tags (R) of DCG-04. Upper right: reaction mechanism of E64d; cysteine protease alkylation.

GBA is degraded in lysosomes by proteases sensitive to inhibition by leupeptin (*N*-acetyl-L-leucyl-L-leucyl-L-argininal), a naturally occurring protease inhibitor produced by actinomycetes (see Scheme 1)⁵⁰. In the eighties several researchers have reported that incubation of cultured cells with leupeptin results in increased levels of GBA as detected with specific antibodies^{36,51}. Augmentation of lysosomal GBA by means of inhibition of lysosomal proteases has not actively been studied as therapeutic approach. Given its potential to increase intralysosomal GBA capacity, we revisited the protection of the enzyme by protease inhibitors. We first demonstrated that also E64d ((2*S*,3*S*)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; Scheme 1) protects GBA against proteolytic degradation and is able to functionally increase GBA capacity in GD lymphoblasts as reflected by a reduction in glucosylsphingosine. Next, we made use of activity-based probes (ABPs) with E64 as scaffold (DCG-04, Scheme 1)^{52,53} to visualize the cysteine proteases potentially involved in GBA breakdown. Based on ABP labeling of cathepsins in fibroblasts and lymphoblasts the most

likely candidates for degrading GBA are cathepsins S, H, B, L and F. *In vitro* degradation of GBA with each of these cathepsins led to breakdown of GBA, although very little with cathepsins S and H. Next, we investigated the role of cathepsins B and F by their knock-out with CRISPR-Cas and that of cathepsin L by knock-down using shRNA. Reduction of neither of these cathepsins was sufficient to increase GBA in cultured cells. Apparently, multiple cathepsins may degrade GBA in lysosomes. The implications of these findings are discussed.

Results

Stabilization of GBA in fibroblasts exposed to leupeptin and E64d.

We first recapitulated the earlier findings by Jonsson et al. that GBA in cultured fibroblasts is protected against degradation by the presence of leupeptin, an inhibitor of serine and cysteine proteases³⁶. For this, fibroblasts homozygous for wildtype and N370S GBA were cultured for 5 days in the presence of 20 μ M leupeptin. The cell lysates were analyzed on GBA content by western blot, labeling of active GBA with ABP MDW933 (Figure S1) and enzyme activity measurement. All three methods showed stabilization of GBA (Figure 1), confirming earlier results³⁶.

Next, we investigated the effect of E64d, a more specific cysteine protease inhibitor. The same stabilizing effect on GBA, visualized with MDW933 labeling and western blot, was induced by the presence of E64d in the culture medium of fibroblasts homozygous for wildtype and N370S GBA as observed for leupeptin (see Figure 2A). An increased enzyme activity in cells treated with E64d was also noted (Figure 2B).

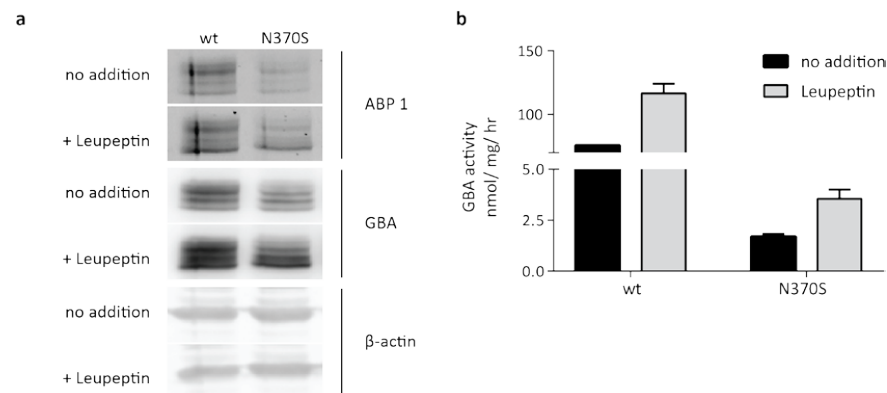


Figure 1. Increased GBA levels in cells cultured with leupeptin. Wildtype and homozygous N370S GBA GD fibroblasts were cultured with or without 20 μ M leupeptin for 5 days. Medium supplemented with or without leupeptin was refreshed on a daily basis. **(a)** Fluorescent labeling of cell lysates with ABP 1 and subsequent western blotting with anti-GBA and anti- β -actin as described in Methods. **(b)** GBA activities measured in the same protein lysates with 4MU- β -D-Glc substrate as described in Methods.

The proteases targeted by E64 have earlier been determined by Greenbaum and colleagues. They synthesized the compound DCG-04 based on E64 as scaffold⁵². DCG-04 has a P2 leucine and was shown to target the same broad set of cysteine proteases as E64. The affinity tag of DCG-04 allowed purification of targeted proteases and their subsequent identification by proteomics. Among the identified proteases were the cathepsins C, S, F, H, K, V, B and L⁵³. We tested the presence of these cathepsins in lysates of fibroblasts using fluorescent DCG-04 as ABP (see Figure 2). Based on apparent molecular weight, cathepsins B, F, H, L, and S were detectable with BODIPY DCG-04 (Figure 2c and d). Subsequently, BODIPY DCG-04 was administered to intact cells and gave rise to increases in GBA (as detected by enzyme activity) comparable to those observed with E64d (see Figure 2e).

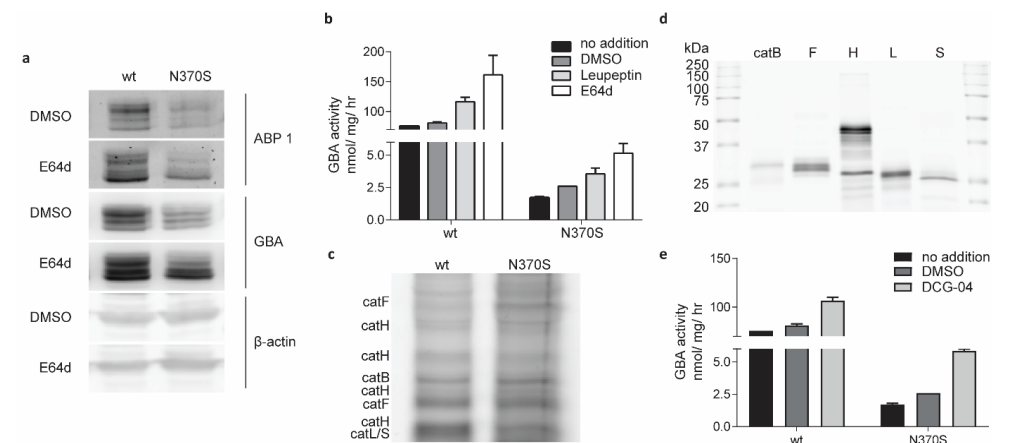


Figure 2. Increased GBA levels in cells cultured with E64d and BODIPY DCG-04. Wildtype and homozygous N370S Gaucher fibroblasts were cultured with or without 20 μ M E64d or 10 μ M BODIPY DCG-04 for 5 days. Medium supplemented with or without E64d or BODIPY DCG-04 was refreshed daily. **(a)** Fluorescent labeling of cell lysates with ABP 1 and subsequent western blotting with anti-GBA and anti- β -actin as described in Methods. **(b)** GBA activities measured in the same protein lysates with 4MU- β -D-Glc substrate as described in Methods. **(c)** Wildtype and N370S fibroblasts labeled with BODIPY DCG-04; cathepsins are indicated based on apparent molecular weight. **(d)** Fluorescent labeling of recombinant cathepsins with BODIPY DCG-04. **(e)** GBA activities measured with 4MU- β -D-Glc substrate in the same control and DMSO treated protein lysates as shown in **(b)**, now compared to cells treated with BODIPY DCG-04.

We subsequently investigated immortalized lymphoblasts of type 1 GD patients, each with at least one N370S *Gba* allele (N370S/N370S; N370S/R463 IVS10; N370S/X, uncharacterized mutation). The presence of E64d in the culture medium for 12 days led to increased active GBA as visualized with ABP ME569 (Figure 3a, 3b and S1). E64d treatment also resulted in

elevated GBA activity (Figure 3c and d). Finally, we determined glucosylsphingosine (GlcSph) in untreated and treated GD lymphoblasts (Figure 3 e and f). E64d treatment led to a decreased GlcSph content of GD lymphoblasts, demonstrating a functional correction in GBA capacity. The lymphoblasts showing the largest increase in GBA activity also showed the largest reduction in GlcSph.

Next, we investigated whether one specific cathepsin among the candidates B, F, H, L and S plays a major role in intralysosomal stability of GBA. The ability of commercial recombinant cathepsins to degrade GBA was determined (Figure 4). For this purpose, we looked into *in vitro* degradation of recombinant (60 kDa) GBA by equal quantities of commercial recombinant cathepsins at acid pH 5.0, mimicking the lysosomal environment. Cathepsin B, F and L generated GBA fragments when 30 ng cathepsin and 250 ng GBA was used (Figure 4).

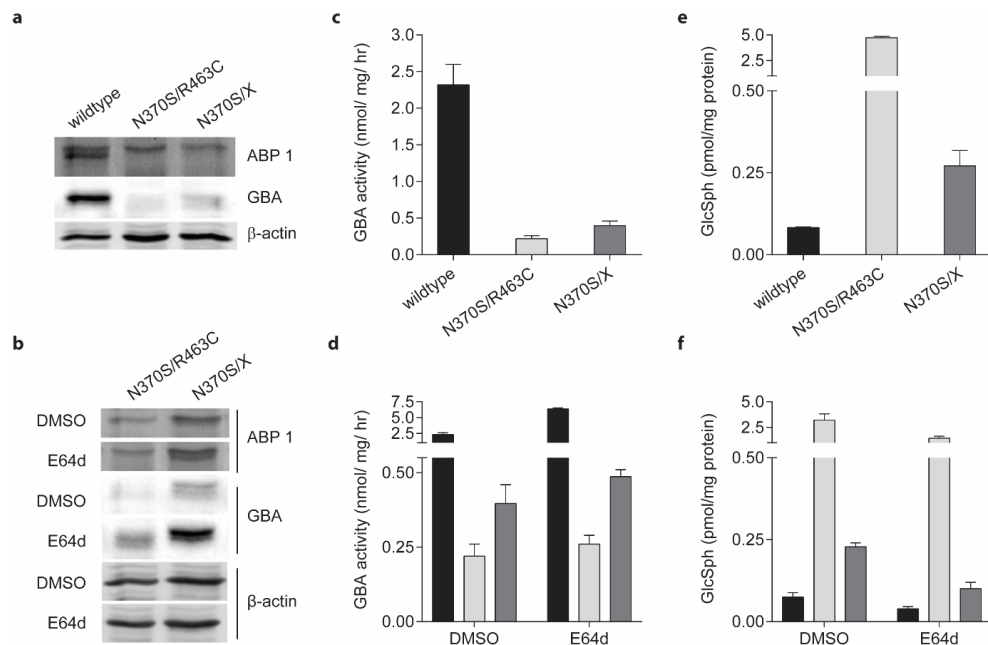


Figure 3. Cathepsin inhibition in lymphoblast cell lines. (a) Active GBA labeling with Cy5 tagged GBA-ABP ME569 in lysates of wildtype and GD lymphoblasts (upper panel), western blotting of GBA (middle panel) and β -actin loading control. (b) Idem to (a), except for cells being prior treated with E64d for 12 days. (c) GBA activity in wildtype and GD lymphoblasts measured by 4MU assay; (d) Idem to (c), except for cells treated with E64d for 12 days. (e) Glucosylsphingosine (GlcSph) in GD lymphoblasts. (f) GlcSph levels in same GD lymphoblasts treated for 12 days with E64d.

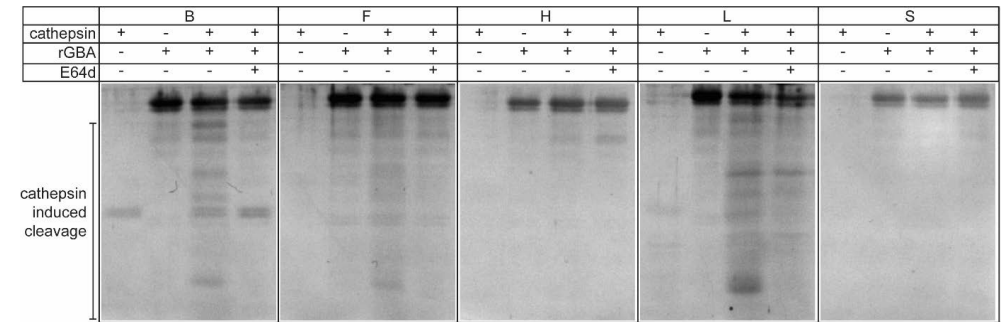


Figure 4. In vitro digestion of pure GBA by recombinant cathepsins. Silver staining of SDS-PAGE gels of recombinant human GBA (250 ng) digested *in vitro* by recombinant cathepsins (30 ng) at pH 5.0 for 1 hour.

Finally, we generated by CRISPR-Cas cells lacking cathepsin B and F. No effect on GBA as detected by ABP labeling or Western blotting was detected in the protease deficient cells (Figure 5a). The generation of cathepsin L-KO cells by CRISPR-Cas was not successful. Therefore, a knockdown in cathepsin L was generated using shRNA (Figure 5b). A major reduction in cathepsin L was obtained, however without effect on cellular GBA level as detected with ABP or Western blotting. Unfortunately, there are no truly specific inhibitors of various cathepsins commercially available to further validate the findings.

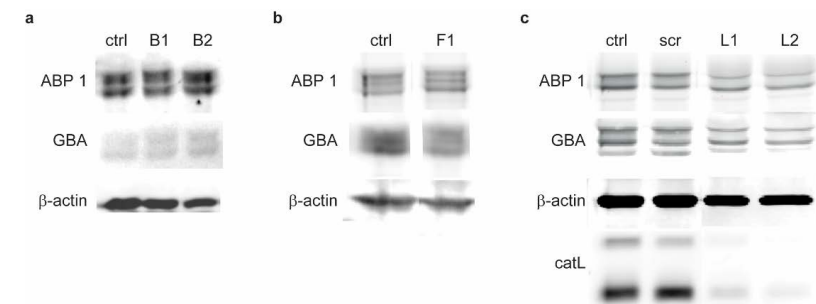
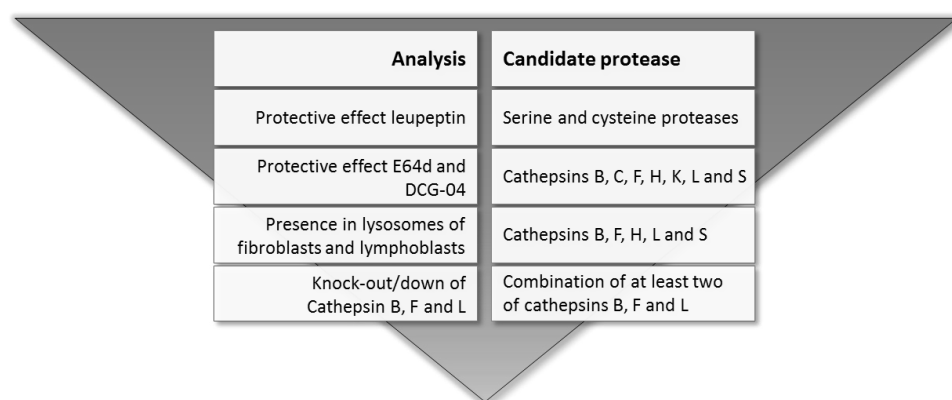


Figure 5. Impact of reduction of cathepsins B, F and L on cellular GBA. (a), (b) A knock-out of cathepsin B and cathepsin F was generated in HEK293T cells by CRISPR-Cas as described in M&M. Cellular GBA was analyzed by ABP labeling and Western blotting with anti-GBA antibody. B1, B2 and F1: CRISPR-Cas clones of cathepsin B and F. (c) Knock-downs of cathepsin L were generated in fibroblast cells using a specific shRNA. Reduction of cathepsin L was determined by Western blotting. Cellular GBA was analyzed by ABP labeling and Western blotting with anti-GBA antibody. Ctrl: untransduced cells, scr: cells transduced with unspecific scrambled shRNA, L1 and L2: generated catL knockdowns 1 and 2.

Discussion

There remains a need for alternative treatments of GD for a variety of reasons. Firstly, the existing ERT fails to prevent neurological manifestations due to poor brain penetrance of the therapeutic enzyme and moreover it is extremely costly and therefore not always accessible. Present substrate reduction therapies are neither able to prevent neuropathology in GD patients. Another need for improving GBA capacity stems from the recent realization that carriers of mutant GBA have a 30-fold increased risk for developing Parkinson disease. Thus, the lysosomal GBA capacity appears to be critical for normal functioning of neuronal cells, in particular motor neurons⁵⁴. It is known that the lysosomal breakdown of GBA is mediated by cysteine and/or serine cathepsins inhibited by leupeptin and is abnormally fast in the case of the common mutant N370S GBA. Of note, cathepsins are found to be increased in plasma as well as tissues of GD patients^{55,56}. We therefore set out to identify which cathepsins are involved in the turnover of GBA with the aim to assess whether it is feasible to significantly increase the half-life of (mutant) GBA in lysosomes by the specific inhibition of one particular cathepsin (see Scheme 2).

Our investigation first revealed that the protective effect on GBA exerted by leupeptin is also observed with E64d and the structurally related DCG-04, inhibitors of multiple cysteine cathepsins, but not serine proteases. E64d and DCG-04 treatment both lead to the desired functional increase of GBA capacity in cultured GD lymphoblasts as reflected by reduction of glucosylsphingosine, the base formed from accumulating GlcCer in lysosomes. Using fluorescent activity-based DCG-04 probes, we next narrowed down the cysteine cathepsins likely involved in lysosomal GBA turnover to the cathepsin B, F, L, H and S. Based on the observed ability of each of these five proteases to digest *in vitro* GBA, the cathepsins B, F and L were considered as prime candidates to mediate lysosomal turnover of GBA. Knockout of cathepsins B and F did not significantly reduce the turnover of GBA in cultured cells. Neither did knockdown of cathepsin L. We therefore conclude that multiple cysteine cathepsins, most likely combinations of cathepsins B, F and L, are able to degrade GBA in lysosomes.



Scheme 2. Candidate proteases involved in lysosomal GBA turnover.

Our findings suggest that in order to increase the half-life of GBA in lysosomes one would need to concomitantly inhibit multiple cysteine cathepsins. Such approach is unattractive for various reasons. Cathepsin F has been reported to influence LIMP-2, the transporter of newly formed GBA to lysosomes⁵⁷. Moreover, cathepsin F deficiency causes Kufs disease type B, characterized by dementia and motor deficits⁵⁸. Cathepsin L is highly expressed in neurons of the cerebral cortex and in Purkinje cells of the cerebellum. It is considered to exert a protective effect, as for example observed in cathepsin B deficient mice that develop lethal hypotrophy and motility defects in the absence of cathepsin L⁵⁹. In conclusion, our study did not reveal a single cathepsin as target to pharmacologically modulate the half-life of GBA in lysosomes and thus boost GBA activity.

Materials & Methods

Materials.

All chemicals used were research grade and obtained from Sigma-Aldrich if not indicated differently. The GBA ABP, MDW933 (cyclophellitol, BODIPY FL)³⁰ as well as BODIPY TMR and Cy5 DCG-04, the ABP for cysteine proteases^{53,60} were synthesized at the Leiden Institute of Chemistry as described previously. For synthesis of GBA ABP ME569 (cyclophellitol, Cy5) see methods. Recombinant human GBA (CerezymeTM) was a gift from Sanofi-Genzyme (Cambridge, MA, USA). Recombinant cathepsin B and S were purchased at EMD Millipore, cathepsin H was from R&D Systems and cathepsins F and L were from Enzo Life Sciences. The fluorogenic substrate 4-methylumbelliferyl- β -glucose was from Glycosynth (Warrington, UK). A SpeedVac Eppendorf concentrator Plus was used during lipid extractions. The antibody for cathepsin L was purchased at Sigma-Aldrich, antibodies for β -actin and GAPDH were from Cell Signaling.

Methods.

Cell culture.

Skin fibroblasts and lymphoblasts of GD patients and controls were obtained after consent and diagnosis was confirmed by genotyping. Lymphoblasts were generated as follows. Leukocytes from healthy donors and from GD patients were isolated by sedimentation on Ficoll-Hypaque density gradients. Mononuclear cells were resuspended in RPMI 1640 medium containing 10% fetal calf serum and EBV viral supernatants (10^7 mononuclear cells/ml) and incubated for 2 h at 37 °C. Cultures were maintained in 25 cm² tissue culture flask for 3 weeks containing 1 μ g/mL cyclosporin A. The cells were washed twice with PBS and then cultured in IMDM medium containing 10 % fetal calf serum and 5 % Pen/Strep (Life Technologies). Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) F12 supplemented with 10 % fetal calf serum, 2 mM L-glutamine and 1 % Pen/Strep (Sigma Aldrich). Fibroblasts were cultured for 5 days and lymphoblasts for 12 days with or without 20 μ M leupeptin or

E64d. E64d is the cell permeable variant of E64. Medium and inhibitors were refreshed daily for fibroblasts and every other day for lymphoblasts. DMSO levels were kept constant between different conditions. Cell lysates were made in K_2HPO_4 - KH_2PO_4 buffer pH 6.5 with 0.1 % Triton X-100.

Lentiviral knockdown and CRISPR knockouts.

Wildtype fibroblast cells transduced with scrambled were infected with non-target shRNA TRC 002. Fibroblast knockdowns of cathepsin L (L1 & L2) were obtained by infection with shRNA TRC 677. CRISPR cathepsin B knockouts were obtained by transfection of HEK293T cells with wildtype cas9 and guideRNA *tcaacaacggaataccacgtgg* (the PAM site indicated in bold). Primers *gatcccatagacacctcagctc*, *tttcaacagtcaggacaatggt*, *gaccacataacagagaggtgctc* and *ccgacatgactcagggtcagg* were used for PCR of relevant genomic cathepsin B fragments. CRISPR cathepsin B clone 1 (B1) was found to contain a 10bp deletion and a 113bp deletion. No other mutations were found, but judging by the frequency with which the 10bp deletion was found, it was concluded that two out of three alleles must have this mutation. Cathepsin B clone 2 (B2) was found to have an allele with a 351 bp deletion, another allele with a 107bp insertion and a third allele with the same 10bp deletion as clone 1. A CRISPR cathepsin F knockout (F1) was obtained by transfection of HEK293T cells with wildtype cas9 and guideRNA *tgtgctgctggcgcgtccgcccggg*. Primers *ccattccccatcaccagagatc* and *acctacttcagagattgctacgc* were used for PCR of cathepsin F fragments. The CRISPR clone for cathepsin F contained a 50bp deletion. No other mutations, nor wildtype sequence were found, so it was assumed that all alleles contained this deletion.

GBA activity measurement.

Activity of GBA was measured with 4-methylumbelliferyl- β -glucoside as substrate in McIlvaine buffer (pH 5.2) containing 0.1 % (v/v) Triton X-100 and 0.2 % (w/v) taurocholate at 37 °C as described earlier⁶¹. Briefly, protein lysates were incubated with substrate for 30 minutes at 37 °C, after which the reaction was stopped with NaOH-glycine (pH 10.3), and fluorescence was measured with a fluorimeter LS55 (Perkin-Elmer, Beaconsfield, UK) at λ_{ex} 366 nm and λ_{em} 445 nm.

Protein determination.

For quantification of protein the Pierce™ BCA Protein Assay Kit (Pierce Biotechnology Inc., No. 23225), was used according to the manufacturer's protocol.

Labeling of cathepsins with fluorescent DCG-04 probe.

Equal amounts of protein in cell lysates were incubated in 50 mM MES buffer (50 mM MES, 50 mM NaCl, 5 mM DTT, 0.0125 % (w/v) digitonin, pH 5.5 with 1 μ M BODIPY TMR or Cy5 DCG-04 for 60 minutes at 37 °C. Of recombinant cathepsins 1 μ g protein was labeled with 1 μ M Cy5 DCG-04 for the same duration at 37 °C.

Labeling of GBA with GBA ABPs

Equal amounts of protein were incubated with 100 nM MDW933 (BODIPY FL) or ME569 (Cy5) for GBA in McIlvaine buffer (pH 5,2) containing 0.1 % triton X-100 as well as 0.2 % taurocholate for 30 minutes at 37 °C.

Gel electrophoresis, fluorescence scanning and Western blotting.

Equal amounts of protein were denatured with 5 \times Laemmli buffer (50 % (v/v) 1 M Tris-HCl, pH 6.8, 50 % (v/v) 100 % glycerol, 8 % (w/v) DTT, 10 % (w/v) SDS, 0.01 % (w/v) bromophenol blue), boiled for 5 minutes at 100 °C and electrophoresed on a 10 % (w/v) SDS-PAGE gel running at 75 V for 30 minutes and afterwards at 150 V. Wet slab-gels were scanned on fluorescence with a Typhoon Variable Mode Imager (Amersham Biosciences) using λ_{EX} 488 nm and λ_{EM} 520 nm (band pass filter 40 nm) for green fluorescent ABP 1 and λ_{EX} 635 nm and λ_{EM} 670 nm (band pass filter 30 nm) for Cy5 labeled GBA ABP. After fluorescence scanning, semi-dry blotting was performed and antigens on blot were visualized by incubation with antibodies as described earlier³⁰. Monoclonal anti-human GBA antibody 8E4 was produced by hybridoma cells as described previously⁶². Scanning of blots was carried out with a Fujifilm LAS-4000 Imager (GE Healthcare Bio-Sciences, Uppsala, Sweden) for ECL and Cy5 fluorescence.

In vitro digestion of recombinant human GBA with recombinant cathepsins.

Recombinant human GBA (Cerezyme™, 250 ng) was incubated with 30 ng recombinant cathepsin B, F, H, L or S in 20 mM MES buffer pH 5.0 containing 150 mM NaCl, 1 mM DTT and 250 mM EDTA. A 40 mM DMSO stock of E64d was diluted into MES buffer to come to a final concentration of 50 μ M in the incubation mixture. Cathepsin alone, GBA alone, GBA with cathepsin, or GBA with cathepsin and E64d was incubated at 37 °C for 60 minutes. Subsequently 5 \times Laemmli buffer was added, samples were boiled and run on a 12.5 % SDS-page gel after which silver staining was performed using the SilverQuest™ Staining Kit from Invitrogen (Carlsbad CA, USA).

Lipid measurements by UPLC-ESI-MS/MS procedures.

GlcSph was determined as described earlier^{63,64}. An internal standard mix (¹³C₅-Sph and ¹³C₅-GlcSph) was added for quantification of the glycosphingoid bases. Briefly, after protein precipitation with $CHCl_3$:MeOH 1:1 (v:v), lipid extraction was performed at the ratio $CHCl_3$:MeOH:H₂O 1:1:0.9 (v:v:v). GlcSph is recovered in the upper phase. The upper phase was dried under N₂ gas stream and the pellet re-dissolved in solution A (10 mM ammonium formate in MeOH). Lipids were measured by UPLC-ESI-MS/MS with an Acquity TQD (Waters Inc.).

Synthesis of ME569 (cyclophellitol Cy5)

Cyclophellitol Cy5 ME569 was synthesized by copper-catalyzed click chemistry of azidocyclophellitol with Cy5 alkyne.

Cyclophellitol Cy5 ME569: Azidocyclophellitol⁶⁵ (24.2 mg, 0.12 mmol) and the desired Cy5-alkyne (84 mg, 0.15 mmol) were dissolved in BuOH/toluene/H₂O (6 mL, 1:1:1, v/v/v). CuSO₄ (0.024 mL, 1 M in H₂O) and sodium ascorbate (0.024 mL, 1 M in H₂O) were added and the reaction mixture was heated at 80 °C for 18 h. Then, the solution was diluted with CH₂Cl₂, washed with H₂O, dried over MgSO₄ and concentrated under reduced pressure. The crude was purified by silica gel column chromatography (CH₂Cl₂ to CH₂Cl₂/MeOH 9:1), subsequently purified by semipreparative reversed-phase HPLC (linear gradient: 45 to 48% B in A, 12 min, solutions used A: 50 mM NH₄HCO₃ in H₂O, B: MeCN) and lyophilized to yield cyclophellitol ME569 as a blue powder (33.5 mg, 44 μmol, 37%). ¹H NMR (400 MHz, CD₃OD): δ 8.25 (t, *J* = 13.1 Hz, 2H), 7.91 (d, *J* = 3.3 Hz, 1H), 7.49 (d, *J* = 7.4 Hz, 2H), 7.44 – 7.39 (m, 2H), 7.31 – 7.24 (m, 4H), 6.63 (t, *J* = 12.4 Hz, 1H), 6.29 (d, *J* = 13.8 Hz, 2H), 4.81 (dd, *J* = 13.9, 3.8 Hz, 1H), 4.61 (dd, *J* = 13.9, 8.6 Hz, 1H), 4.42 (s, 2H), 4.09 (t, *J* = 7.4 Hz, 2H), 3.63 (s, 3H), 3.60 (d, *J* = 8.0 Hz, 1H), 3.24 – 3.20 (m, 1H), 3.12 (t, *J* = 14.9 Hz, 1H), 3.02 – 3.00 (m, 1H), 2.41 – 2.35 (m, 1H), 2.25 (t, *J* = 7.3 Hz, 2H), 1.91 (s, 6H), 1.85 – 1.78 (m, 2H), 1.72 (s, 12H), 1.50 – 1.43 (m, 2H) ppm; ¹³C NMR (101 MHz, CD₃OD): δ 175.8, 175.4, 174.6, 155.5, 155.5, 146.2, 144.2, 143.5, 142.6, 142.5, 129.8, 129.7, 126.7, 126.2, 126.2, 125.2, 123.4, 123.3, 112.0, 111.8, 104.5, 104.3, 78.2, 72.5, 68.6, 57.6, 55.5, 50.8, 50.5, 50.5, 44.8, 44.6, 36.5, 35.6, 31.6, 28.1, 28.0, 27.3, 26.4 ppm; HRMS: calcd. for C₄₂H₅₃N₆O₅ [M]⁺ 721.4072, found: 721.4070.

References

1. E. Beutler, G. A. G. in *C.R. Scriver, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, 8th ed.* 3635–3668 (McGraw-Hill, New York, 2001).
2. Brady, R. O., Kanfer, J. N., Bradley, R. M. & Shapiro, D. Demonstration of a deficiency of glucocerebrosidase-cleaving enzyme in Gaucher's disease. *J. Clin. Invest.* **45**, 1112–1115 (1966).
3. Patrick, A. A deficiency of glucocerebrosidase in Gaucher's disease. *Biochem J.* **97**, 17C–18C (1965).
4. Hruska, K. S., LaMarca, M. E., Scott, C. R. & Sidransky, E. Gaucher disease: mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA). *Hum. Mutat.* **29**, 567–583 (2008).
5. Kolter, T. & Sandhoff, K. Lysosomal degradation of membrane lipids. *FEBS Lett.* **584**, 1700–1712 (2010).
6. Vaccaro, A. M. *et al.* Saposin C mutations in Gaucher disease patients resulting in lysosomal lipid accumulation, saposin C deficiency, but normal prosaposin processing and sorting. *Hum. Mol. Genet.* **19**, 2987–2997 (2010).
7. Marques, A. R. A. *et al.* Glucosylated cholesterol in mammalian cells and tissues: formation and degradation by multiple cellular β-glucosidases. *J. Lipid Res.* **57**, 451–463 (2016).
8. Bussink, A. P., van Eijk, M., Renkema, G. H., Aerts, J. M. & Boot, R. G. The biology of the Gaucher cell: the cradle of human chitinases. *Int. Rev. Cytol.* **252**, 71–128 (2006).
9. Boven, L. A. *et al.* Gaucher cells demonstrate a distinct macrophage phenotype and resemble alternatively activated macrophages. *Am. J. Clin. Pathol.* **122**, 359–69 (2004).
10. Ferraz, M. J. *et al.* Gaucher disease and Fabry disease: New markers and insights in pathophysiology for two distinct glycosphingolipidoses. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **1841**, 811–825 (2014).
11. Ferraz, M. J. *et al.* Lysosomal glycosphingolipid catabolism by acid ceramidase: formation of glycosphingoid bases during deficiency of glycosidases. *FEBS Lett.* **590**, 716–725 (2016).
12. Dekker, N. *et al.* Elevated plasma glucosylsphingosine in Gaucher disease: relation to phenotype, storage cell markers, and therapeutic response. *Blood* **118**, e118–e127 (2011).
13. Boot, R. G. *et al.* Marked elevation of the chemokine CCL18/PARC in Gaucher disease: a novel surrogate marker for assessing therapeutic intervention. *Blood* **103**, 33–9 (2004).
14. Kramer, G. *et al.* Elevation of glycoprotein nonmetastatic melanoma protein B in type 1 Gaucher disease patients and mouse models. *FEBS Open Bio* **6**, 902–913 (2016).
15. van Dussen, L. *et al.* Value of plasma chitotriosidase to assess non-neuronopathic Gaucher disease severity and progression in the era of enzyme replacement therapy. *J. Inherit. Metab. Dis.* **37**, 991–1001 (2014).
16. Schoonhoven, A. *et al.* Monitoring of Gaucher patients with a novel chitotriosidase assay. *Clin. Chim. Acta.* **381**, 136–9 (2007).
17. Møller, H. J. *et al.* Soluble CD163: a marker molecule for monocyte/macrophage activity in disease. *Scand. J. Clin. Lab. Invest. Suppl.* **237**, 29–33 (2002).
18. Boot, R. G. *et al.* Glucocerebrosidase genotype of Gaucher patients in The Netherlands: limitations in prognostic value. *Hum. Mutat.* **10**, 348–58 (1997).
19. Aerts, J. M., Hollak, C., Boot, R. & Groener, A. Biochemistry of glycosphingolipid storage disorders: implications for therapeutic intervention. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **358**, 905–14 (2003).
20. Biegstraaten, M. *et al.* A monozygotic twin pair with highly discordant Gaucher phenotypes. *Blood Cells. Mol. Dis.* **46**, 39–41 (2011).
21. Lachmann, R. H., Grant, I. R., Halsall, D. & Cox, T. M. Twin pairs showing discordance of phenotype in adult Gaucher's disease. *QJM* **97**, 199–204 (2004).
22. Ohashi, T. *et al.* Characterization of human glucocerebrosidase from different mutant alleles. *J. Biol. Chem.* **266**, 3661–7 (1991).
23. van Weely, S. *et al.* Role of pH in determining the cell-type-specific residual activity of glucocerebrosidase in type 1 Gaucher disease. *J. Clin. Invest.* **91**, 1167–75 (1993).
24. Schmitz, M., Alfalah, M., Aerts, J. M. F. G., Naim, H. Y. & Zimmer, K.-P. Impaired trafficking of mutants of lysosomal glucocerebrosidase in Gaucher's disease. *Int. J. Biochem. Cell Biol.* **37**, 2310–20 (2005).
25. Dvir, H. *et al.* X-ray structure of human acid-β-glucosidase, the defective enzyme in Gaucher disease. *EMBO Rep.* **4**, 704–709 (2003).
26. Brumshtein, B. *et al.* Cyclodextrin-mediated crystallization of acid β-glucosidase in complex with amphiphilic bicyclic nojirimycin analogues. *Org. Biomol. Chem.* **9**, 4160 (2011).
27. Rempel, B. P. & Withers, S. G. Covalent inhibitors of glycosidases and their applications in biochemistry and biology. *Glycobiology* **18**, 570–586 (2008).
28. Kallemeijn, W. W. *et al.* A Sensitive Gel-based Method Combining Distinct Cyclophellitol-based Probes for the Identification of Acid/Base Residues in Human Retaining -Glucosidases. *J. Biol. Chem.* **289**, 35351–35362 (2014).

29. Witte, M. D., van der Marel, G. a, Aerts, J. M. F. G. & Overkleeft, H. S. Irreversible inhibitors and activity-based probes as research tools in chemical glycobiology. *Org. Biomol. Chem.* **9**, 5908–5926 (2011).
30. Witte, M. D. *et al.* Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nat. Chem. Biol.* **6**, 907–913 (2010).
31. Kallemeijn, W. W. *et al.* Novel activity-based probes for broad-spectrum profiling of retaining β -exoglucosidases in situ and in vivo. *Angew. Chemie - Int. Ed.* **51**, 12529–12533 (2012).
32. Aerts, J. M. *et al.* Glucocerebrosidase, a lysosomal enzyme that does not undergo oligosaccharide phosphorylation. *Biochim. Biophys. Acta* **964**, 303–8 (1988).
33. Reczek, D. *et al.* LIMP-2 Is a Receptor for Lysosomal Mannose-6-Phosphate-Independent Targeting of β -Glucocerebrosidase. *Cell* **131**, 770–783 (2007).
34. Rothaug, M. *et al.* LIMP-2 expression is critical for β -glucocerebrosidase activity and α -synuclein clearance. *Proc. Natl. Acad. Sci.* **111**, 15573–15578 (2014).
35. Gaspar, P. *et al.* Action myoclonus-renal failure syndrome: diagnostic applications of activity-based probes and lipid analysis. *J. Lipid Res.* **55**, 138–145 (2014).
36. Jonsson, L. M. V *et al.* Biosynthesis and maturation of glucocerebrosidase in Gaucher fibroblasts. *Eur. J. Biochem.* **164**, 171–179 (1987).
37. Aerts, J. M. *et al.* Comparative study on glucocerebrosidase in spleens from patients with Gaucher disease. *Biochem. J.* **269**, 93–100 (1990).
38. Van Weely, S. *et al.* Function of oligosaccharide modification in glucocerebrosidase, a membrane-associated lysosomal hydrolase. *Eur. J. Biochem.* **191**, 669–77 (1990).
39. Barton, N. W. *et al.* Replacement therapy for inherited enzyme deficiency--macrophage-targeted glucocerebrosidase for Gaucher's disease. *N. Engl. J. Med.* **324**, 1464–70 (1991).
40. Smid, B. E. *et al.* Biochemical response to substrate reduction therapy versus enzyme replacement therapy in Gaucher disease type 1 patients. *Orphanet J. Rare Dis.* **11**, 28 (2016).
41. Aerts, J. M. F. G., Yasothan, U. & Kirkpatrick, P. Velaglucerase alfa. *Nat. Rev. Drug Discov.* **9**, 837–8 (2010).
42. Zimran, A. *et al.* Pivotal trial with plant cell-expressed recombinant glucocerebrosidase, taliglucerase alfa, a novel enzyme replacement therapy for Gaucher disease. *Blood* **118**, 5767–73 (2011).
43. Desnick, R. J. & Schuchman, E. H. Enzyme Replacement Therapy for Lysosomal Diseases: Lessons from 20 Years of Experience and Remaining Challenges. *Annu. Rev. Genomics Hum. Genet.* **13**, 307–335 (2012).
44. Platt, F. M. *et al.* Inhibition of substrate synthesis as a strategy for glycolipid lysosomal storage disease therapy. *J. Inherit. Metab. Dis.* **24**, 275–90 (2001).
45. Cox, T. *et al.* Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. *Lancet* **355**, 1481–1485 (2000).
46. Cox, T. M. *et al.* Eliglustat compared with imiglucerase in patients with Gaucher's disease type 1 stabilised on enzyme replacement therapy: a phase 3, randomised, open-label, non-inferiority trial. *Lancet* **385**, 2355–2362 (2015).
47. Steet, R. *et al.* Selective action of the iminosugar isofagomine, a pharmacological chaperone for mutant forms of acid-beta-glucosidase. *Biochem. Pharmacol.* **73**, 1376–83 (2007).
48. Benito, J. M., García Fernández, J. M. & Ortiz Mellet, C. Pharmacological chaperone therapy for Gaucher disease: a patent review. *Expert Opin. Ther. Pat.* **21**, 885–903 (2011).
49. Siebert, M., Sidransky, E. & Westbroek, W. Glucocerebrosidase is shaking up the synucleinopathies. *Brain* **137**, 1304–1322 (2014).
50. Hozumi, M., Ogawa, M., Sugimura, T., Takeuchi, T. & Umezawa, H. Inhibition of tumorigenesis in mouse skin by leupeptin, a protease inhibitor from Actinomycetes. *Cancer Res.* **32**, 1725–8 (1972).
51. Sun, Y., Qi, X. & Grabowski, G. A. Saposin C is required for normal resistance of acid beta-glucosidase to proteolytic degradation. *J. Biol. Chem.* **278**, 31918–23 (2003).
52. Greenbaum, D., Medzihradsky, K. F., Burlingame, A. & Bogyo, M. Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. *Chem. Biol.* **7**, 569–581 (2000).
53. Greenbaum, D. *et al.* Chemical approaches for functionally probing the proteome. *Mol. Cell. Proteomics* **1**, 60–8 (2002).
54. Aflaki, E., Westbroek, W. & Sidransky, E. The Complicated Relationship between Gaucher Disease and Parkinsonism: Insights from a Rare Disease. *Neuron* **93**, 737–746 (2017).
55. Moran, M. T. *et al.* Pathologic gene expression in Gaucher disease: up-regulation of cysteine proteinases including osteoclastic cathepsin K. *Blood* **96**, 1969–1978 (2000).
56. Vitner, E. B. *et al.* Altered expression and distribution of cathepsins in neuronopathic forms of Gaucher disease and in other sphingolipidoses. *Hum. Mol. Genet.* **19**, 3583–90 (2010).
57. Zunke, F. *et al.* Characterization of the complex formed by β -glucocerebrosidase and the lysosomal integral membrane protein type-2. *Proc. Natl. Acad. Sci.* **113**, 3791–3796 (2016).
58. Smith, K. R. *et al.* Cathepsin F mutations cause Type B Kufs disease, an adult-onset neuronal ceroid lipofuscinosis. *Hum. Mol. Genet.* **22**, 1417–23 (2013).
59. Sevenich, L., Pennacchio, L. A., Peters, C. & Reinheckel, T. Human cathepsin L rescues the neurodegeneration and lethality in cathepsin B/L double-deficient mice. *Biol. Chem.* **387**, 885–91 (2006).
60. Hillaert, U. *et al.* Receptor-Mediated Targeting of Cathepsins in Professional Antigen Presenting Cells. *Angew. Chemie Int. Ed.* **48**, 1629–1632 (2009).
61. Aerts, J. M. *et al.* The occurrence of two immunologically distinguishable beta-glucocerebrosidases in human spleen. *Eur. J. Biochem.* **150**, 565–74 (1985).
62. Aerts, J. M. *et al.* A procedure for the rapid purification in high yield of human glucocerebrosidase using immunoaffinity chromatography with monoclonal antibodies. *Anal. Biochem.* **154**, 655–63 (1986).
63. Mirzaian, M. *et al.* Mass spectrometric quantification of glucosylsphingosine in plasma and urine of type 1 Gaucher patients using an isotope standard. *Blood Cells, Mol. Dis.* **54**, 307–314 (2015).
64. Mirzaian, M. *et al.* Simultaneous quantitation of sphingoid bases by UPLC-ESI-MS/MS with identical ¹³C-encoded internal standards. *Clin. Chim. Acta* **466**, 178–184 (2017).
65. Li, K.-Y. *et al.* Synthesis of Cyclophellitol, Cyclophellitol Aziridine, and Their Tagged Derivatives. *European J. Org. Chem.* **2014**, 6030–6043 (2014).

Supplemental Figures

Table S1. Cathepsin gene expression and protein levels in human Gaucher spleens versus control spleens. Gene expression of proteases was determined via qPCR and statistical significance was determined by unpaired t-tests. Protein levels were analyzed via quantification of Western Blots of spleen tissue lysates.

	Gene expression		Protein
	Fold change GD vs Wt	Significance	Fold change GD vs Wt
catA	2.9	0,0091	0.63
catB	2.1	n.s.	4.21
catC	1.2	n.s.	1.27
catD	11.4	0,0035	1.65
catF	3.1	n.s.	0.43
catH	0.8	n.s.	5.90
catK	47.3	0,045	0.77
catL	1.9	n.s.	2.12
catS	8.2	0,0089	0.76
catW	0.2	n.s.	1.19
catZ	3.8	0,004	3.28
LGMN	4.6	0,0081	0.92

Decreased compared to Wt
Increased compared to Wt

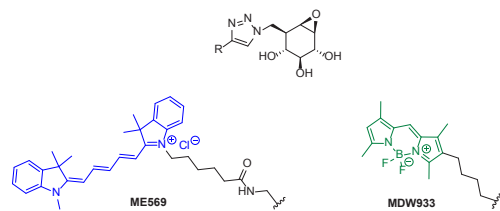


Figure S1. Chemical structures of GBA ABPs used in this work.

Figure S2. Labeling of active cysteine cathepsins in spleen homogenates at pH5.5 using BODIPY TMR DCG-04 ABP. CBB: Coomassie Brilliant Blue staining.

