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

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

7

β-Xylosidase and transxylosidase activities of human glucocerebrosidase

β-Xylosidase and transxylosidase activities of human glucocerebrosidase

Based on

Mina Mirzaian[1*], Maria J. Ferraz[1*], Daphne E.C. Boer[1], Saskia V. Ousoren[1], Marc Hazeu[1], Jasper Wermink[1], Per Haberkant[1#], Sybrin P. Schroder[3], Karen Ghauharali[2], Edward Blommaart[2], Roelof Ottenhoff[2], Andre R.A. Marques[1], Rianne Meijer[1], Wouter Kallemeijn[1], Rolf G. Boot[1], Herman S. Overkleeft[3], Navraj S. Pannu[4], Johannes M. Aerts[1]

[1]Dept. of Medical Biochemistry, LIC, Leiden University
[2]Dept. of Medical Biochemistry, AMC, Amsterdam
[3]Dept. of Bio-organic Synthesis, LIC, Leiden University
[4]Dept. of Macromolecular Biochemistry, LIC, Leiden University
#Present address; EMBL, Heidelberg
[*]These authors contributed equally to this work

Manuscript pending submission

Abstract

The lysosomal retaining β-glucosidase, glucocerebrosidase (GBA1), hydrolyzes β-glucosidic substrates and transglucosylates cholesterol to cholesterol-β-glucoside (GlcChol). We here present evidence that the enzyme also cleaves 4-methylumbelliferyl-β-D-xylose (4MU-β-Xyl) *in vitro* and *in vivo*. This activity is stimulated by saposin C. In addition, GBA1 is shown to transxylosylate fluorescent 25-NBD-cholesterol and natural cholesterol using 4MU-β-Xyl as donor. The xylosyl-cholesterol (XylChol) formed by GBA1 acts as subsequent acceptor to render di-xylosyl-cholesterol. Exposure of GBA1 to cholesterol and sequentially 4MU-β-Glc and 4MU-β-Xyl results in generation of GlcXylChol. The cytosolic β-glucosidase GBA3 shows *in vitro* also β-xylosidase and transxylosylase activitiy, in contrast to the membrane-bound cellular β-glucosidase GBA2. Cultured cells also generate xylosylated cholesterols when exposed to 4MU-β-Xyl in their medium, independent of the presence of glucosylceramide synthase.. This synthesis is enhanced by the drug U18666A causing an increase in lysosomal cholesterol. The prior inhibition of GBA1 with conduritol B-epoxide prohibits generation of xylosylated cholesterols by cells. In conclusion, our findings reveal further catalytic versatility of GBA1. The natural occurrence of xylosylated cholesterol as well as other xylosylated lipids warrants future investigation.

Introduction

Xylose mimics glucose except for the lacking pendant CH_2OH group. It is a main building block of the ubiquitous xylan in plants[1]. In animals, xylose is present in O-glycans of proteoglycans as the first saccharide linked to serine or threonine residues. It is added by the protein by UDP-xylose dependent xylosyltransferases, an essential step in the synthesis of the glycosaminoglycans heparan sulfate, keratan sulfate and chondroitin sulfate[2]. The human body synthesizes UDP-xylose is from UDP-glucuronate by UDP-glucuronic acid decarboxylase 1 encoded by the *UXS1* gene[3]. Since the studies by Fisher & Kent and Patel & Tappel, degradation of β-xylosides in animals is thought to depend on β-glucosidases[4,5]. We earlier reported that the lysosomal acid β-glucosidase GBA1, *aka* glucocerebrosidase, can hydrolyze 4-methylumbelliferyl-β-xyloside (4MU-β-Xyl)[6]. Inherited deficiency of GBA1 causes Gaucher disease (GD), a lysosomal disorder characterized by the glucosylceramide (GlcCer) accumulating macrophages in tissues[7,8]. The occurrence of storage of β-D-xylose-containing glycopeptides in GD patients has not been actively investigated. More recently another catalytic activity of GBA1 has been recognized. Through transglucosylation, GBA1 can transfer of glucose from β-glucoside substrates to cholesterol, thus forming β-D-glucosylcholesterol (GlcChol)[9,10]. Glew and colleagues earlier demonstrated that GBA1 can transglucosylate retinol *in vitro*[11]. Formation of GlcChol by GBA1 also takes place *in vivo*[10]. In Niemann Pick type C disease (NPC), cholesterol accumulates in lysosomes is due to genetic defects in either NPC1 or NPC2, two proteins mediating the export of the sterol from lysosomes[12]. In NPC, GBA1 actively generates GlcChol[10]. The generation of GlcChol by cells can also be induced by exposure to U18666A, an inhibitor of cholesterol export from lysosomes. The transglucosylation in these cells is prohibited by inhibition of GBA1[10]. Also GBA2, the β-glucosidase inserted in the cytoplasmic leaflet of membranes, shows transglucosylase activity *in vitro* and *in vivo*[10,13–15].

We here examined whether GBA1 is also able to generate xylosyl-β-D-cholesterol (XylChol). It is reported that GBA1 indeed xylosylates cholesterol, generating not only XylChol but even di-xylosyl-cholesterol (Xyl_2Chol) and traces of tri-xylosyl-cholesterol (Xyl_3Chol). The physiological relevance of these findings is discussed.

Results

Cleavage of 4-methylumbelliferyl-β-D-xylose by GBA1.

We first incubated pure recombinant hGBA1 with either 4-methylumbelliferyl β-D-xylose (4MU-β-Xyl) or 4-methylumbelliferyl β-D-glucose (4MU-β-Glc). The enzyme was found to release fluorescent 4MU from both substrates. The activity towards 4MU-β-Xyl was about 50-fold less due to a higher Km and lower Vmax (Table 1). The pH optimum of the activity of GBA1 towards both substrates was found to be similar (Fig 1A). The same held true for the stimulation of activity by taurocholate (fig 1B). The activity towards both substrates was also comparably stimulated by recombinantly produced saposin C (Fig 1C). The kcat/Km of

recombinant GBA1 is about 40-fold higher for 4MU-β-Glc than 4MU-β-Xyl (Table 1). GBA1 uses double displacement for catalysis with E340 as nucleophile and E325 as acid/base[16]. Blocking E340 through permanent linkage of the suicide inhibitor cyclophellitol eliminates activity[17]. The activities of GBA1 towards 4MU-β-Glc and 4MU-β-Xyl substrates were both inhibited by pre-incubation for 90 minutes and subsequent activity assay for 60 minutes. The slightly higher apparent IC_{50} with 4MU-β-Glc (85 nM) than with 4MU-β-Xyl (61 nM) is likely due to greater protection by the β-D-glucose substrate against irreversible inhibition of GBA1. A recently synthesized xylose analogue of cyclophellitol[18] also irreversibly inactivated GBA1, although with lower affinity than cyclophellitol (Table 1). The apparent IC_{50} with 4MU-β-Glc (10.16 µM) was slightly higher than with 4MU-β-Xyl substrate (6.41 µM), presumably again due to better protection by the former substrate against irreversible inhibition of GBA1.

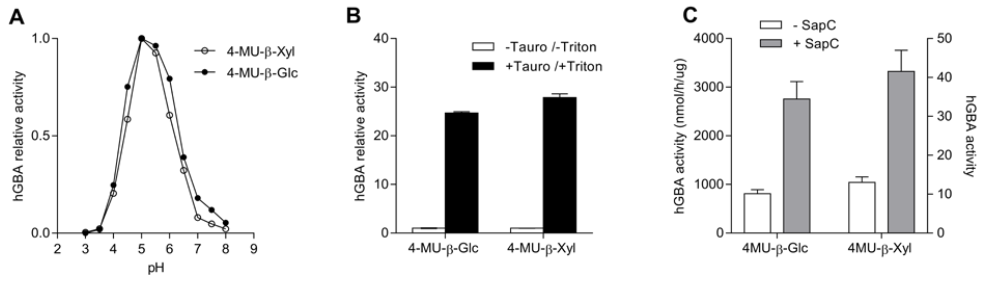


Figure 1. Cleavage of 4MU-β-Glc and 4MU-β-Xyl by recombinant hGBA1. A. pH optimum of 4MU release by GBA1 from 4MU-β-Glc (closed circles) and 4MU-β-Xyl (open circles); **B.** Stimulation by 0.2 % (w/v) taurocholate of 4MU release from the substrates 4MU-β-Glc (left) and 4MU-βXyl (right). The measured activity is expressed as 100 % in the absence of taurocholate at pH 5.2, with 0.1 % (v/v) Triton X-100; **C.** Stimulation of 4MU release from the substrates 4MU-β-Glc (left axis) and 4MU-β-Xyl (right axis) by recombinant saposin C at pH 4.5 in the presence of phosphatidylserine.

Table 1. Kinetic parameters hGBA1.

hGBA1	*4MU-β-Glc*	*4MU-β-Xyl*
IC50 cyclophellitol (µM)	0.085 ± 0.002	0.061 ± 0.002
IC50 D-xylo-cyclophellitol (µM)	10.16 ± 1.03	6.41 ± 0.47
Km (mM)	0.76 ± 0.06	5.24 ± 1.04
Vmax (nmol/h.mg)	$1.23 \times 10^6 \pm 3.32 \times 10^4$	$1.88 \times 10^5 \pm 2.88 \times 10^4$
Kcat/Km (mM/s)	25.03	0.55

Transxylosylase activity of GBA1.

We next studied the potential transxylosidase activity of recombinant GBA1. Enzyme was incubated for 16 hours with 4MU-β-Glc or 4MU-β-Xyl as donors and fluorescent 25-NBD-cholesterol as acceptor. Products were visualized by HPTLC and fluorescence scanning. Formation of fluorescent sterol metabolites occurred with both donors (fig 2A). With 4MU-β-D-Glc, glucosylated 25-NBD-cholesterol is formed as earlier described[10]. In the case of 4MU-β-Xyl, two novel fluorescent metabolites were detected, presumably mono- and di-xylosylated 25-NBD cholesterol.

Subsequently we used natural cholesterol as acceptor in the same assay and the formed products were analyzed by LC-MS/MS. Formation of XylChol, Xyl_2Chol, and traces of Xyl_3Chol was detected (fig 2B). In sharp contrast, incubation of GBA1 and cholesterol with 4MU-β-Glc only yields GlcChol as product (fig 2C).

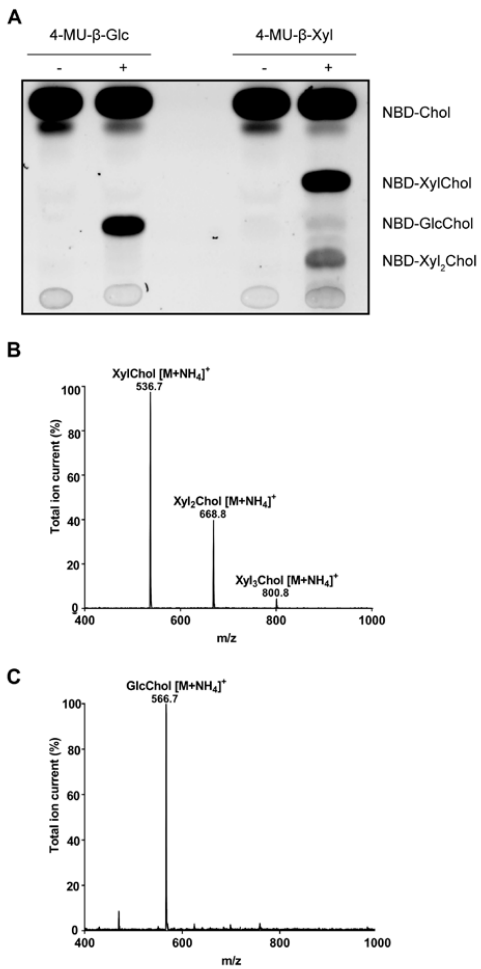


Figure 2. Transxylosylation and transglucosylation of cholesterol by GBA1. A. HPTLC analysis of fluorescent products formed from 25-NBD-cholesterol, following incubation with GBA1 in the presence of 4MU-β-Glc or 4MU-β-Xyl for 16 h; **B.** LC-MS/MS analysis of products formed during 1 h incubation of GBA1, cholesterol and 4MU-β-Xyl; **C.** LC-MS/MS analysis of products formed during 1 h incubation of GBA1, cholesterol and 4MU-β-Glc.

Time dependence of glycosidase and transglycosidase activities of GBA1.

GBA1 and cholesterol were incubated with 4MU-β-Glc or 4MU-β-Xyl for different time periods at 37 ˚C. The release of fluorescent 4MU and formation of glycosylated products was determined. The generated GlcChol was already maximal after 30 min incubation and then declined with time (fig 3A). This indicates that the formed GlcChol is also subject to hydrolysis by GBA1. In sharp contrast, XylChol showed no reduction over time, and Xyl_2Chol was formed after a lag period (fig 3B). Apparently, XylChol is barely hydrolyzed and serves as acceptor for further xylosylation. Even Xyl_2Chol may act as acceptor, rendering Xyl_3Chol (fig 3). Comparison of the release of 4MU with concomitant formation of glycosylated sterol indicates that GBA1 shows considerably higher net transxylosylation than transglucosylation efficiency (fig 3).

We next studied the outcome of the sequential incubation of GBA1 and cholesterol with 4MU-β-Xyl (3h) followed by 4MU-β-Glc (1h). Formation of GlcXylChol (with m/z 698.5 > 369.3) was demonstrable at these conditions, again indicating that XylChol is as excellent acceptor in glycosylation reaction (Supplemental Table 2).

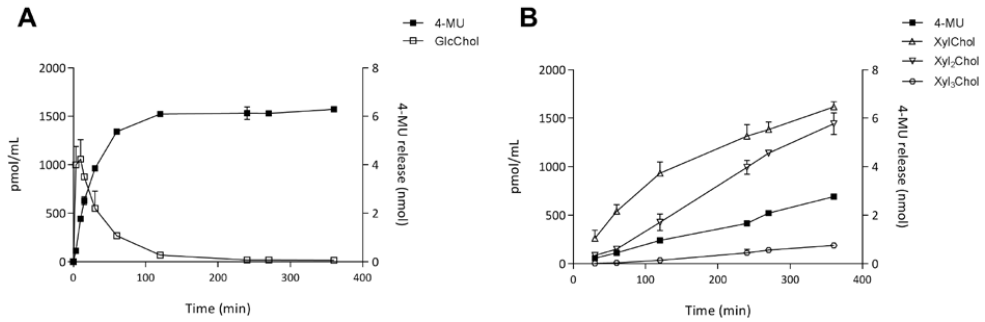


Figure 3. Glycosylation of cholesterol in time. A. Release of 4MU from 4MU-β-Glc and concomitant formation of glucosylated cholesterol; **B.** Release of 4MU from 4MU-β-Xyl and concomitant formation of xylosylated cholesterol. GBA1 was incubated at pH 5.2 in the presence of taurocholate and Triton X-100 with 3.0 mM 4MU-substrates for the indicated times after which released 4MU and formed glycosylated cholesterol were determined.

In vivo formation of xylosylated cholesterol.

Building on the *in vitro* findings, potential transxylosylation by cultured RAW264.7 cells exposed to 3.7 mM 4MU-β-Xyl in the medium was studied. Cells were incubated without or with conduritol β-epoxide (CBE), an irreversible GBA1 inhibitor, either in the absence (fig. 4A) or presence of 10 µM U18666A (fig. 4B) to induce lysosomal accumulation of cholesterol[10]. Formation of XylChol, Xyl_2Chol and Xyl_3Chol was determined by LC-MS/MS (fig. 4A). The levels of xylosylated cholesterols were markedly increased by exposure of cells to U18666A and prohibited by pre-incubation with CBE (fig. 4A and B).

We next examined the possible involvement of UDP-glucose dependent glucosylceramide synthase (GCS)[19,20] in the formation of xylosylated cholesterols. HEK293 cells deficient in GCS produced XylChol on a par to corresponding *wt* cells when exposed to 4MU-β-Xyl and U18666A (fig. 4C and D).

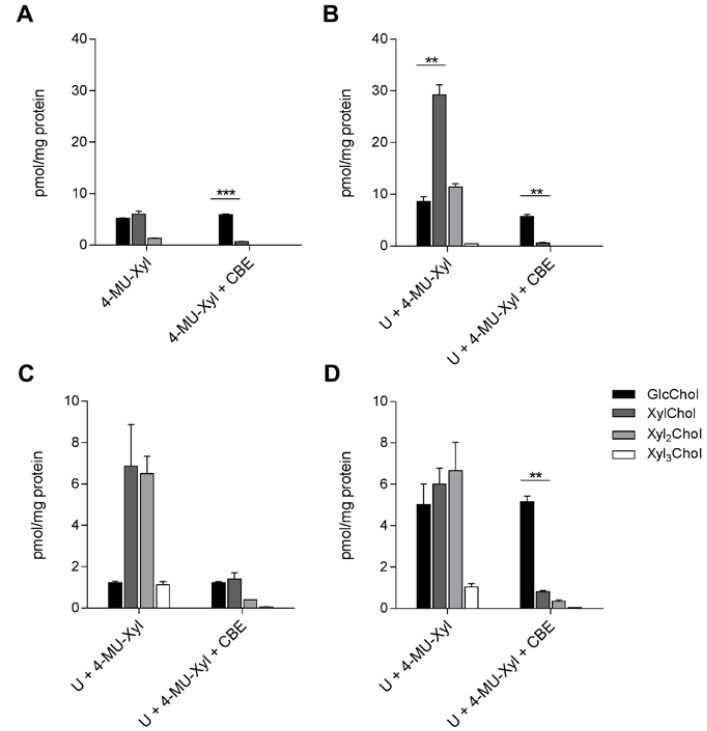


Figure 4. *In vivo* formation of xylosylated cholesterols. A. LC-MS/MS detection of formed xylosylated cholesterols in RAW264.7 cells incubated with 3.7 mM 4MU-β-Xyl for 24 h, with or without CBE; **B.** Idem, in the presence of 10 µM U18666A; **C.** LC-MS/MS detection of formed xylosylated and glucosylated cholesterols in HEK293 exposed to 3.7 mM 4MU-β-Xyl and U18666A for 24 hours in the presence or absence of CBE; **D.** LC-MS/MS detection of formed glycosylated cholesterols in HEK293 cells deficient in GCS as in panel C. Significance of differences in formation of GlcChol and XylChol was determined and is shown in the panels A-D.

Specificity of transxylosylation.

Finally, we investigated the potential transxylosylation by the two other human retaining β-glucosidases, GBA2 and GBA3. We previously noticed that GBA2, but not GBA3, can mediate

transfer of the glucosyl moiety from 4MU-β-D-glucose to cholesterol or ceramide[10]. While this finding was recapitulated (fig. 5), no xylosylation by GBA2 was concomitantly detectable, consistent with its inability to hydrolyze 4MU-β-D-Xyl[21]. However, GBA3, albeit less prominent than GBA1, is able to hydrolyze 4MU-β-Xyl as well as to transxylosylate cholesterol (fig. 5).

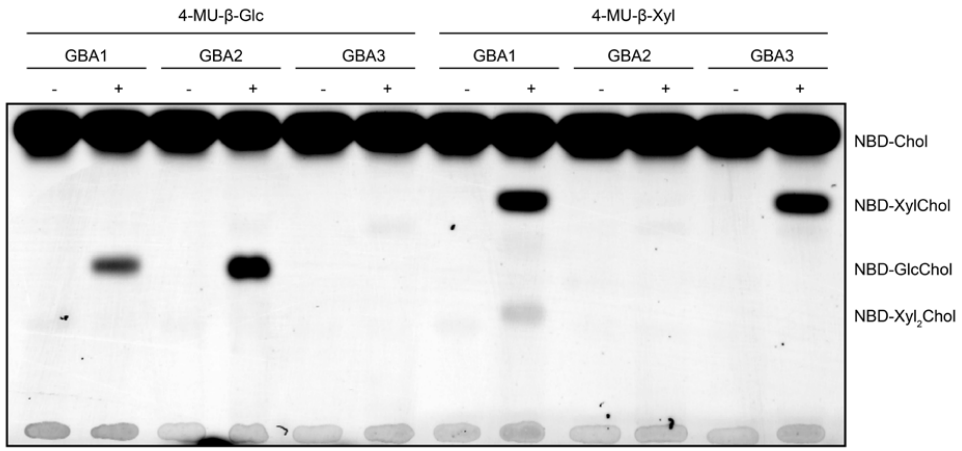


Figure 5. Lack of transxylosylation by GBA2. HPTLC analysis of formation of glycosylated 25-NBD-cholesterol by β-glucosidases with 4MU-β-Xyl and 4MU-β-Glc as donor. Enzymes: recombinant hGBA1; lysate of HEK293 cells overexpressing GBA2; lysate of HEK293 cells overexpressing GBA3. Incubation for 16 hours with (+) or without (-) enzyme preparation.

7

Discussion

Our present investigation reveals further catalytic versatility of the lysosomal glucocerebrosidase GBA1. The enzyme *in vitro* cleaves besides 4MU-β-Glc also 4MU-β-Xyl. Moreover, GBA1 uses both substrates as sugar donors in transglycosylation of cholesterol. Next, GBA1 in cultured cells generates xylosylated cholesterol when exposed to 4MU-β-Xyl. The induction of lysosomal cholesterol accumulation with U18666A increases formation of xylosylated cholesterols, a reaction prohibited by inactivation of GBA1 with the irreversible inhibitor CBE. Both *in vitro* and *in vivo,* GBA1 may even produce di-xylosyl-cholesterol through a repetitive transglucosylation not seen with 4MU-β-Glc as sugar donor[10]. The affinity of GBA1 for 4MU-β-Glc as substrate for cleavage is higher than that for 4MU-β-Xyl. Likewise, XylChol is a poorer substrate for hydrolysis by GBA1 than GlcChol. Following exposure of GBA1 to cholesterol and 4MU-β-Xyl, the concentration of XylChol steadily builds up and it starts to act as acceptor in a second round of transxylosylation, rendering Xyl_2Chol. Incubation of GBA1 and cholesterol with a mixture of 4MU-β-Xyl and 4MU-β-Glc leads to

formation of GlcXylChol, further illustrating the suitability of XylChol as acceptor in transglycosylation.

Net formation of XylChol by GBA1 exceeds that of GlcChol, a phenomenon that can be ascribed to the lower rate of hydrolysis of XylChol than GlcChol. At present, it can't be excluded that a xylose covalently bound to the catalytic nucleophile E340 of GBA1 is also somehow transferred more efficiently to cholesterol than a covalently bound glucose. Further insight might be obtained by crystallography employing β-glucose and β-xylose configured cyclophellitol-epoxides covalently bound to E340-GBA1. Of note, Aerts and co-workers earlier reported relative higher transxylosylase than transglucosylase efficiency of a β-D-glucosidase from *Stachybotrys atra*[19], quite comparable to our findings with GBA1.

The physiological relevance of transxylosylation catalyzed by GBA1 is presently unclear. Mass spectrometry suggests the presence of XylChol (m/z 536.5 > 369.3) in liver of $Npc1^{-/-}$ mice (data not shown). Of note, in the same livers accumulation of GlcChol has previously been detected[10]. Definitive confirmation of XylChol in NPC liver by NMR analysis of purified lipid is needed. In this connection, a key question is whether physiological xyloside donors occur. Several β-xylosidic compounds are known to be produced by plants and their uptake via food can a priori not be excluded[20]. β-D-xylosyl moieties are also present in endogenous proteoglycans from which xylosyl-peptides are formed during lysosomal degradation. It is unclear whether these are suitable donors for GBA1-mediated formation of xylosylated sterols. In theory, a UDP-xylose dependent xylosyltransferase may hitherto generate unknown β-xyloside donors to be used in transxylosylation. One such candidate is UGT3A2, a UDP-xylose-utilizing glycosyltransferase reported to glycosylate various hydrophobic structures *in vitro*[22]. Lastly, it should be rigorously investigated whether other lipids besides cholesterol may act as acceptors in transxylosylation by GBA1[10]. We already observed that GBA1 *in vitro* also transxylosylates sphingosine and ceramide (not shown).

Our investigation has further revealed that GBA3, a cytosolic glucosidase implicated in metabolism of xenophobic glycosides[23], also shows xylosidase and transxylosidase activity. In contrast, the β-glucosidase GBA2, recently shown to be a potent transglucosylase[10], has no significant activity towards β-xyloside substrates. Apparently the pendant CH_2OH group in glucoside substrates is crucial in the interaction of GBA2 with substrate. The importance of the presence of the additional CH_2OH group in glucose is also suggested by the much lower affinity of GBA2 for the inhibitor conduritol-B-epoxide as compared to cyclophellitol (with the pendant CH_2OH group).

In conclusion, human GBA1 is more versatile in catalysis as hitherto considered. Investigation of the (patho)physiological relevance of various reactions catalyzed by GBA1 might increase our understanding of the complex symptomatology of Gaucher disease[7,8] and other conditions for which abnormal GBA1 imposes a risk such as multiple myeloma and α-synucleinopathies like Parkinsonism and Lewy-body dementia[24,25].

7

Materials and Methods

Materials

25-[N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol (25-NBD-Cholesterol) and cholesterol were from Avanti Polar Lipids (Alabaster, AL, USA). 4-methylumbelliferyl-β-D-glucose (4MU-β-Glc) and 4-methylumbelliferyl-β-D-xylose (4MU-β-Xyl) were purchased from Glycosynth™ (Winwick Quay Warrington, Cheshire, England). Conduritol β epoxide (D, L-1,2-anhydro-*myo*-inositol; CBE) was from Enzo Life Sciences Inc. (Farmingdale, NY, USA). Cholesterol trafficking inhibitor U18666A, 1-*O*-cholesteryl-β-D-glucose (β-cholesteryl glucose, β-GlcChol) and ammonium formate (LC-MS quality) were from Sigma-Aldrich (St Louis, MO, USA). GBA1 inhibitor cyclophellitol, and the GBA2 inhibitor N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin (AMP-DNM) and GBA3 inhibitor α-1-C-nonyl-DIc (anDIX) were synthesized at Leiden Institute of Chemistry (Leiden, The Netherlands)[23,26]. Cerezyme®, a recombinant human GBA1 was obtained from Genzyme (Genzyme Nederland, Naarden, The Netherlands). LC-MS–grade methanol, 2-propanol, water, and HPLC-grade chloroform was purchased from Biosolve. D-xylo-cyclophellitol was synthesized as described previously[18].

Mouse materials

Npc1$^{-/-}$ mice (*Npc1*^{nih}) as well as wild-type littermates (*Npc1*$^{+/+}$), were generated by crossing *Npc1*$^{+/-}$ males and females. The heterozygous BALB/c Nctr-*Npc1*^{m1N}/J mice (stock number 003092) were obtained from the Jackson Laboratory (Bar Harbor, USA). Mouse pups were genotyped according to published protocols[27]. Mice (± 3 weeks old) received the rodent AM-II diet (Arie Blok Diervoeders, Woerden, The Netherlands). The mice were housed at the Institute Animal Core Facility in a temperature- and humidity-controlled room with a 12-h light/dark cycle and given free access to food and water *ad libitum*. All animal protocols were approved by the Institutional Animal Welfare Committee of the Academic Medical Centre Amsterdam in the Netherlands (DBC101698). Animals were first anesthetized with a dose of Hypnorm (0.315 mg/mL phenyl citrate and 10 mg/mL fluanisone) and Dormicum (5 mg/mL midazolam) according to their weight. The given dose was 80 µL/10 g bodyweight. Animals were sacrificed by means of cervical dislocation. Organs were collected by surgery, rinsed with PBS, directly snap-frozen in liquid nitrogen and stored at -80 °C. Later, homogenates were made from the frozen material in 25 mM potassium phosphate buffer pH 6.5, supplemented with 0.1 % (v/v) Triton X-100 and protease inhibitors (4 µL of buffer per mg of tissue).

Cloning and expression of cDNAs encoding β-glucosidases GBA2 and GBA3.

Stable GBA2 expressing HEK293T cells were generated as follows. The PCR-amplified human GBA2 (GBA2 acc. nr: NM_020944.2) coding sequence (using the following oligonucleotides: sense. 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCACCATGGGGACCCAGGATCCAG-3'and antisense 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTCACTCTGGGCTCAGGTTTG-3') was cloned into pDNOR-221 and sub-cloned in pLenti6.3/TO/V5-DEST using the Gateway system (Invitrogen). Correctness of the construct was verified by sequencing. To produce lentiviral particles HEK293T cells were transfected with pLenti6.3-GBA2 in combination with the envelope and packaging plasmids pMD2G, pRRE and pRSV. Subsequently, culture supernatant containing viral particles was collected and used for infection of HEK293T cells. Selection by blasticidin for several weeks rendered cells stably expressing human GBA2 as determined by activity assays. For stable expression of human GBA3 in HEK293T cells, the PCR-amplified GBA3 (GBA3 acc. Nr: NM_020973.4) coding sequence (using the following oligonucleotides: sense. 5'-GAATTCG CCGCCACCATGGCTTTCCCTGCAGGATTTG-3' and antisense 5'- GCGGCCGCAGATGTG CTTCAAGGCCATTG-3') was cloned in pcDNA3.1/Zeo and transfected into HEK293T cells using FuGENE® 6 Transfection Reagent (Promega Benelux, Leiden, The Netherlands). Zeocin selection for several weeks rendered cells stably expressing human GBA3 as confirmed by activity assays.

In vitro assays with fluorogenic 4-methylumbelliferyl-β-D-glycosides.

Enzymatic activity of GBA1 was measured with 3.7 mM 4MU-β-Glc or 4MU-β-Xyl, dissolved in 150 mM McIlvaine buffer (pH 5.2 supplemented with 0.2 % (w/v) sodium taurocholate, 0.1 % (v/v) Triton X-100) and 0.1 % BSA[28]. 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_{ex} 445 nm. Enzymatic activity of GBA2 was measured in lysates of cells overexpressing the enzyme using the same conditions as above, but without detergents and at pH 5.8. Enzymatic activity of GBA3 was measured without detergents at pH 7.0[23].

Stimulation of GBA1 activity by the activator protein saposin C, produced recombinantly in *E. coli*[29], was monitored with 3.7 mM 4-MU-β-glucoside as substrate in 150mM McIlvaine buffer pH4.5 (150 mM McIlvaine buffer (pH 4.5) containing 0.1 % (w/v) bovine serum albumin and 0.4 mg/ml phosphatidylserine[30].

In vitro assay of transglycosylase activity with fluorescent 25-NBD-Cholesterol as acceptor.

Lysates of COS-7 cells overexpressing GBA2, and GBA3, and recombinant GBA1 were used to determine transglycosylase activity of every enzyme. The assays were performed as described earlier[10]. First, 40 µL of homogenate of cells overexpressing GBA2, or GBA3 was pre-incubated with 10 µL of 25 µM CBE in water for 20 min (samples containing diluted recombinant GBA1 were pre-incubated in the absence of CBE). To every sample 200 µL of the appropriate buffer containing 100 µM of donor (either 4MU-β-Xyl or 4MU-β-Glc) and 40 µM of acceptor (either 25-NBD-Cholesterol), was added. Transglycosylase activity of GBA2 overexpressing cells was measured in 150 mM McIlvaine buffer pH 5.8 and the assay for recombinant GBA1 was performed in 150 mM McIlvaine buffer pH 5.2 containing 0.1 % BSA, 0.1 % Triton X-100 and 0.2 % sodium taurocholate. For GBA3 the assay contained 100 mM HEPES buffer, pH 7.0. After 16 h of incubation at 37 °C, the reaction was ended by addition of chloroform/methanol (1:1, v/v) and lipids were extracted according to Bligh and Dyer[31]. Afterwards lipids were separated by thin layer chromatography on HPTLC silica gel 60 plates

(Merck, Darmstadt, Germany) using chloroform/methanol (85:15, v/v) as eluent followed by detection of NBD-labelled lipids using a Typhoon Variable Mode Imager (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA)[21].

***In vitro* assay of transglycosylase activity with cholesterol as acceptor.**
Assays with natural cholesterol as acceptor were performed exactly as described in the section above and the subsequent analysis of products was performed by LC-MS/MS as described in the section below. In short, pure recombinant GBA1 (IU) was incubated at 37 ˚C with 32 µM cholesterol and 3.7 mM 4MU-β-Xyl and/or 4MU-β-Glc in 150 mM McIlvaine buffer pH 5.2 containing 0.1 % BSA, 0.1 % Triton X-100 and 0.2 % sodium taurocholate for the indicated time periods. The incubations were stopped by addition of chloroform/methanol (1:1, v/v) and lipids were extracted according to Bligh and Dyer.

Assays with cultured RAW264.7 and HEK293 cells.
Experiments with cultured RAW264.7 and HEK293 cells exposed to 3.7 mM 4MU-β-Xyl or 4MU-β-Glc in the medium, either with or without U18666A (10 µM), inducing lysosomal cholesterol accumulation, were performed as described earlier[10]. Lysosomal GBA1 was irreversibly inhibited by prior incubation of cells with 300 µM conduritol β-epoxide (CBE). Cells were harvested and lipids extracted as earlier described[10]. In HEK293 cells glucosylceramide synthase (GCS)[32] was knocked-down by the CRISPR-Cas method.

Analysis of GlyChol and XylChol by LC-MS/MS.
A Waters Xevo-TQS micro instrument was used in all experiments. The instrument consisted of a UPLC system combined with a tandem quadruple mass spectrometer as mass analyzer. Data were analyzed with Masslynx 4.1 Software (Waters, Milford MA, USA). Tuning conditions for both GlcChol and XylChol's in ES^+ (electrospray positive) mode are presented in Supplemental table 1. GlcChol, $^{13}C_6$-GlcChol and XylChol's were separated using a BEH C18 reversed-phase column (2.1 x 50 mm, particle size 1.7 µm; Waters), by applying an isocratic elution of mobile phases, 2-propanol:H_2O 90:10 (v/v) containing 10 mM ammonium formate (Eluent A) and methanol containing 10 mM ammonium formate (Eluent B). The column temperature and the temperature of the auto sampler were kept at 23 °C and 10 °C respectively during the run. The flow rate was 0.25 mL/min. Volume of injection was 10 µL.

For the identification of a new compound of interest, the extracted sample was dried and dissolved in 100 µL of Eluent B. The sample was introduced in the mass spectrometer using LC-MS (0 to 5.5 min to the detector). MS parents scan and daughters scan were performed (figure 2). As for GlcChol[10], the most abundant species of XylChol are ammonium adducts, $[M+NH_4]^+$ and the product ion 369.3 represents the cholesterol part of the molecule after loss of the xylose moiety. Ammonium adducts of XylChol, Xyl_2Chol and Xyl_3Chol show the transitions 536.5>369.3, 668.5>369.3 and 800.5>369.3 respectively. The transitions for GlcChol are 566.5>369.3 and for $^{13}C_6$-GlcChol 572.5>369.3.

For Multiple Reaction Monitoring (MRM) the UPLC program was applied during 5.5 minutes consisting of 10 % eluent A and 90 % eluent B. The divert valve of the mass spectrometer was programmed to discard the UPLC effluent before (0 to 0.8 min) and after (4.5 to 5.5 min) the elution of the analytes to prevent system contamination. The retention time of both GlcChol and the internal standard $^{13}C_6$-GlcChol was 1.36 min. XylChol's were generated *in vitro* by incubation of GBA1 with cholesterol. The retention time of XylChol, Xyl_2Chol and Xyl_3Chol was 1.71 min, 1.49 min and 1.40 min respectively (Supplemental figure 1).

LC-MS/MS quantitation of GlcChol and XylChol's produced *in vitro.*
Following incubation of GBA1 and cholesterol with either 4MU-β-Glc or 4MU-β-Xyl, from 180 µL of sample, to which 12.5 pmol of $^{13}C_6$-GlcChol in methanol was added, lipids were extracted according to the Bligh and Dyer method by addition of methanol, chloroform and water (1:1:0.9, v/v/v). The lower phase was taken to dryness in an Eppendorf concentrator. Isolated lipids were purified by water/butanol extraction (1:1, v/v). The upper phase (butanol phase) was taken to the dryness. The residue is dissolved in 100 µL eluent B, sonicated in bath sonicator and samples were analyzed by LC-MS.

LC-MS/MS quantitation of GlcChol and XylChol's in cultured cells.
Cells were homogenized in water by sonication on ice. The protein concentration was around 1 mg/ml. Prior to extraction, 12.5 pmol of ^{13}C-labelled GlcChol in methanol (used as an internal standard) was added to 100 µL of homogenate. After protein precipitation, the supernatant was further treated as described above.

Protein determination.
Protein was analysed with the Pierce BCA Protein Assay kit (Thermo Scientific). Absorbance was measured in EL808 Ultra Microplate Reader (BIO-TEK Instruments Inc.) at 562 nm.

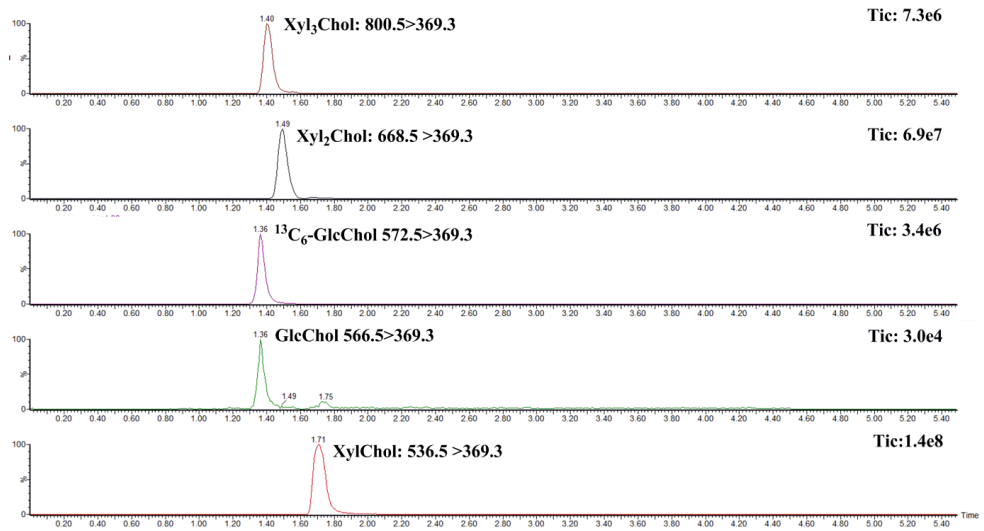
Statistical Analysis.
Values in figures are presented as a mean ± S.D. Data were analyzed by unpaired Student's t-test. *P* values < 0.05 were considered significant. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

References

1. Rennie, E. A. & Scheller, H. V. Xylan biosynthesis. *Curr. Opin. Biotechnol.* **26,** 100–7 (2014).
2. Esko, J. D., Kimata, K. & Lindahl, U. *Proteoglycans and Sulfated Glycosaminoglycans*. *Essentials of Glycobiology* (2009).
3. Moriarity, J. L. *et al.* UDP-glucuronate decarboxylase, a key enzyme in proteoglycan synthesis: cloning, characterization, and localization. *J. Biol. Chem.* **277,** 16968–75 (2002).

4. Fisher, D. & Kent, P. W. Rat liver beta-xylosidase, a lysosomal membrane enzyme. *Biochem. J.* **115,** 50P–51P (1969).
5. Patel, V. & Tappel, A. L. Identity of beta-glucosidase and beta-xylosidase activities in rat liver lysosomes. *Biochim. Biophys. Acta* **191,** 86–94 (1969).
6. van Weely, S., Brandsma, M., Strijland, a, Tager, J. M. & Aerts, J. M. Demonstration of the existence of a second, non-lysosomal glucocerebrosidase that is not deficient in Gaucher disease. *Biochim. Biophys. Acta* **1181,** 55–62 (1993).
7. Beutler, E. & Grabowski, G. A. in *The Metabolic and Molecular Bases of Inherited Disease. 7th ed. New York, NY: McGraw-Hill;* (ed. McGraw-Hill) 2641–2670 (1995).
8. 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).
9. Akiyama, H., Kobayashi, S., Hirabayashi, Y. & Murakami-Murofushi, K. Cholesterol glucosylation is catalyzed by transglucosylation reaction of β-glucosidase 1. *Biochem. Biophys. Res. Commun.* **441,** 838–43 (2013).
10. 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).
11. Vanderjagt, D. J., Fry, D. E. & Glew, R. H. Human glucocerebrosidase catalyses transglucosylation between glucocerebroside and retinol. *Biochem. J.* 309–15 (1994).
12. Vanier, M. T. Complex lipid trafficking in Niemann-Pick disease type C. *J. Inherit. Metab. Dis.* **38,** 187–99 (2015).
13. Yildiz, Y. *et al.* Mutation of β-glucosidase 2 causes glycolipid storage disease and impaired male fertility. **116,** (2006).
14. Boot, R. G. *et al.* Identification of the non-lysosomal glucosylceramidase as beta-glucosidase 2. *J. Biol. Chem.* **282,** 1305–12 (2007).
15. Körschen, H. G. *et al.* The non-lysosomal β-glucosidase GBA2 is a non-integral membrane-associated protein at the endoplasmic reticulum (ER) and Golgi. *J. Biol. Chem.* **288,** 3381–93 (2013).
16. 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).
17. Witte, M. D. *et al.* Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nat. Chem. Biol.* **6,** 907–913 (2010).
18. Schröder, S. P. *et al.* A Divergent Synthesis of l - arabino - and d -xylo -Configured Cyclophellitol Epoxides and Aziridines. *European J. Org. Chem.* **2016,** 4787–4794 (2016).
19. Aerts, G. M., Van Opstal, O. & De Bruyne, C. K. β-d-glucosidase-catalysed transfer of the glycosyl group from aryl β-d-gluco- and β-d-xylo-pyranosides to phenols. *Carbohydr. Res.* **100,** 221–233 (1982).
20. Kay, C. D., Mazza, G., Holub, B. J. & Wang, J. Anthocyanin metabolites in human urine and serum. *Br. J. Nutr.* **91,** 933–42 (2004).
21. van Weely, S., Brandsma, M., Strijland, A., Tager, J. M. & Aerts, J. M. Demonstration of the existence of a second, non-lysosomal glucocerebrosidase that is not deficient in Gaucher disease. *Biochim. Biophys. Acta* **1181,** 55–62 (1993).
22. Mackenzie, P. I. *et al.* Identification of UDP glycosyltransferase 3A1 as a UDP N-acetylglucosaminyltransferase. *J. Biol. Chem.* **283,** 36205–10 (2008).
23. Dekker, N. *et al.* The cytosolic β-glucosidase GBA3 does not influence type 1 Gaucher disease manifestation. *Blood Cells, Mol. Dis.* **46,** 19–26 (2011).
24. Westbroek, W. *et al.* A new glucocerebrosidase-deficient neuronal cell model provides a tool to probe pathophysiology and therapeutics for Gaucher disease. *Dis. Model. Mech.* **9,** 769–78 (2016).
25. Nair, S. *et al.* Clonal Immunoglobulin against Lysolipids in the Origin of Myeloma. *N. Engl. J. Med.* **374,** 555–561 (2016).
26. Overkleeft, H. S. *et al.* Generation of specific deoxynojirimycin-type inhibitors of the non-lysosomal glucosylceramidase. *J. Biol. Chem.* **273,** 26522–7 (1998).
27. Marques, A. R. A. *et al.* Reducing GBA2 Activity Ameliorates Neuropathology in Niemann-Pick Type C Mice. *PLoS One* **10,** e0135889 (2015).
28. Aerts, J. M. *et al.* The occurrence of two immunologically distinguishable beta-glucocerebrosidases in human spleen. *Eur. J. Biochem.* **150,** 565–74 (1985).
29. Ahn, V. E., Leyko, P., Alattia, J.-R., Chen, L. & Privé, G. G. Crystal structures of saposins A and C. *Protein Sci.* **15,** 1849–57 (2006).
30. Aerts, J. M. *et al.* Conditions affecting the activity of glucocerebrosidase purified from spleens of control subjects and patients with type 1 Gaucher disease. *Biochim. Biophys. Acta* **1041,** 55–63 (1990).
31. Bligh, E. G. & Dyer, W. J. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Can. J. Biochem. Physiol.* **37,** 911–917 (1959).
32. Ichikawa, S., Sakiyama, H., Suzuki, G., Hidari, K. I. & Hirabayashi, Y. Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyzes the first glycosylation step of glycosphingolipid synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 12654 (1996).

Supplemental data



Supplemental Figure 1. Chromatogram of XylChol, Xyl_2Chol, Xyl_3Chol, GlcChol and $^{13}C_6$-GlcChol.

Supplemental Table 1. MS/MS instrument parameters.

Capillary voltage	**3.50 KV**
Cone voltage	20 V
Source temperature	150 °C
Desolvation temperature	450 °C
Cone gas	50 L/h
Desolvation gas	950 L/h
Collision voltage	15 V
Type	Multiple reaction monitoring
Ion mode	ES^+ (electrospray positive)
Dwell time	0.1 s
Interchannel delay	0.005 s
Interscan delay	0.005 s
Transitions:	**RT (min.):**
GlcChol	1.36
$^{13}C_6$-GlcChol	1.36
XylChol	1.71
Xyl_2Chol	1.49
tXyl_3Chol	1.40
Fit weight	None
Smooth method	Mean
Smooth width	2

7

Supplemental Table 2. Formation of hybrid GlcXylChol following incubation of GBA1 with 1:1 mixture of 4MU-β-Xyl and 4MU-β-Glc.

MRM transitions	*4MU-Xyl (3h) 4MU-Glc (1h)*	*pmol/mL*
536.5 > 369.3	XylChol	631.56
566.5> 369.3	GlcChol	293.20
668.5> 369.3	Xyl2Chol	76.34
698.5> 369.3	XylGlcChol	10.58
800.5> 369.3	Xyl3Chol	3.34
830.5> 369.3	Xyl2GlcChol	0.42