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2,3-Di-*O*-tetradecyl-1-*O*-(β-D-glucopyranosyl)-*sn*-glycerol is a substrate for human glucocerebrosidase

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Glucocerebrosidase, the lysosomal enzyme that is deficient in patients with Gaucher's disease, hydrolyses non-physiological aryl β-D-glucosides and glucocerebroside, its substrate *in vivo*. We document that 2,3,-di-*O*-tetradecyl-1-*O*-(β-D-glucopyranosyl)-*sn*-glycerol (2,3,-di-14:0-β-Glc-DAG) inhibits human placental glucocerebrosidase activity *in vitro* (K_i 0.18 mM), and the nature of inhibition is typical of a mixed-type pattern. Furthermore, 2,3-di-14:0-β-Glc-DAG was shown to be an excellent substrate for the lysosomal β-glucosidase (K_m 0.15 mM; $V_{max.}$ 19.8 units/mg) when compared with the natural substrate glucocerebroside (K_m 0.080 mM; $V_{max.}$ 10.4 units/mg). The observations that (i) glucocerebrosidase-catalysed hydrolysis of 2,3-di-14:0-β-Glc-DAG is inhibited by conduritol B epoxide and glucosylsphingosine, and (ii) spleen and brain extracts from patients with Gaucher's disease are unable to hydrolyse 2,3-di-14:0-β-Glc-DAG demonstrate that the same active site on the enzyme is responsible for catalysing the hydrolysis of 4-methylumbelliferyl β-D-glucopyranoside, glucocerebroside and 2,3-di-14:0-β-Glc-DAG. With the aid of computer modelling we have established that the oxygen atoms in 2,3-DAG-Glc at the C-1, C-4*, C-5* (the ring oxygen in glucose) and C-2 positions correspond topologically to the oxygens at C-1, C-4* and C-5* and the nitrogen atom attached to C-2 respectively in glucocerebroside (* signifies a carbon atom in glucose); furthermore, all of the distances with respect to overlap of corresponding heteroatoms range from 0.02 Å to 0.77 Å (0.002–0.077 nm). A root-mean-square deviation of 0.31 Å (0.031 nm) was obtained when the energy-minimized structures of 2,3-di-14:0-β-Glc-DAG and glucocerebroside were compared using the latter four heteroatom co-ordinates.

INTRODUCTION

Glucocerebrosidase (glucosylceramidase, EC 3.2.1.45), a lysosomal membrane-bound β-glucosidase, is responsible for the breakdown of glucocerebroside (Fig. 1*a*) to glucose and ceramide *in vivo* in mammals; it can also efficiently catalyse the hydrolysis of non-physiological aryl β-D-glucosides such as 4-methylumbelliferyl β-D-glucoside (MUGlc) and *p*-nitrophenyl β-D-glucoside [1]. The enzyme is of considerable medical interest owing to its profound deficiency in the lysosomal storage disorder, Gaucher's disease [2,3]. The molecular basis for the enzymic deficit has been attributed to the presence of single-base mutations in the gene encoding the enzyme [4–6].

Included among the neutral glycoglycerolipids are the glycosylated diacyl-, alkylacyl- and dialkyl-glycerols. Whereas diacyl compounds occur as membrane constituents of some micro-organisms and in the chloroplast membrane of higher plants, glycosylated alkylacylglycerols are found in mammalian testis, human saliva and tissues of the central nervous system [7]. Because of the marked physical and chemical similarities of glucosyldiacyl (or dialkyl) glycerols with glucocerebroside, it occurred to us that 2,3-di-*O*-tetradecyl-1-*O*-(β-D-glucopyranosyl)-*sn*-glycerol (2,3-di-14:0-β-Glc-DAG, Fig. 1*b*) might be a substrate for the lysosomal β-glucosidase. In this paper, we demonstrate that the amphipathic 2,3-di-14:0-β-Glc-DAG is an excellent substrate for human placental glucocerebrosidase. We also examined tissue extracts from patients with Gaucher's disease in an effort to determine whether the mutation that is causal for decreasing catalytic activity towards glucocerebroside also abolishes the ability to hydrolyse 2,3-di-14:0-β-Glc-DAG.

MATERIALS AND METHODS

MUGlc, ATP, $NADP^+$, sodium taurodeoxycholate (TDC), sphingosyl β-D-glucopyranoside (glucosylsphingosine), β-glucosidase (from almonds), hexokinase and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Conduritol B epoxide (CBE) was obtained from BioMol Research Labs, Philadelphia, PA, U.S.A. Penta-*O*-acetyl-α/β-D-glucose, penta-*O*-acetyl-α/β-D-galactose and 2,3-isopropylidene-*sn*-glycerol were obtained from Pfanstiehl Laboratories, Waukegan, IL, U.S.A. The solvents used for organic synthesis were of reagent grade and were redistilled under anhydrous conditions as reported elsewhere [8,9]. All other reagents used were of the highest grade available.

2,3-Di-*O*-tetradecyl-*sn*-glycerol was synthesized from 2,3-*O*-isopropylidene-*sn*-glycerol via 1-*O*-benzyl-*sn*-glycerol, as described elsewhere for the 1,2-*sn*- enantiomer [8]. The pure chiral ditetradecylglycerol was then condensed with the appropriate acetobromosugars, prepared by treatment of the sugar peracetate with anhydrous 30% HBr/acetic acid (BDH, Poole, Dorset, U.K.), in the presence of an equimolar mixture of mercuric bromide and mercuric cyanide using a 1:1 (v/v) mixture

Abbreviations used: 2,3-di-14:0-β-Glc-DAG, 2,3-di-*O*-tetradecyl-1-*O*-(β-D-glucopyranosyl)-*sn*-glycerol; 2,3-di-14:0-β-Gal-DAG, 2,3-di-*O*-tetradecyl-1-*O*-(β-D-galactopyranosyl)-*sn*-glycerol; TDC, sodium taurodeoxycholate; MUGlc, 4-methylumbelliferyl β-D-glucopyranoside; CBE, conduritol B epoxide; L_α; lamellar liquid crystalline phase; L_β, lamellar gel phase.

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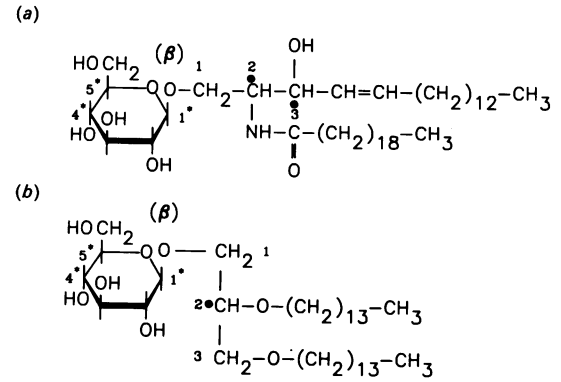

Fig. 1. Structures of glucocerebroside (*a*) and 2,3-di-14:0-β-Glc-DAG (*b*)

The black circles adjacent to the carbon atoms indicate chiral centres in the molecules. The asterisk is included in the numbering of carbon atoms in glucose to differentiate them from the carbon atoms in the sphingosine and glycerol moieties.

of dry acetonitrile and dichloroethane containing anhydrous $CaSO_4$ [8,9]. The resulting condensation product was filtered through glass wool and purified on a silica gel column. Elution with hexane/ethyl acetate (12:1, v/v) gave the α-anomer followed by the β-anomer, which crystallized from ethanol. The acetylated intermediate was deprotected by treatment with triethylamine/methanol/water (2:4:1, by vol.) at approx. 50 °C. The resulting 2,3-ditetradecyl-1-*O*-(β-D-glucopyranosyl)-*sn*-glycerol was purified on a silica gel column using a chloroform/acetonitrile gradient. The lipid was then recrystallized, first from acetone and then from methanol. All analytical data were in agreement with the expected structures. Calorimetric studies of aqueous dispersions of the 2,3-di-14:0-β-Glc-DAG are consistent with a high level of purity and display three thermal events on heating at 32, 49, and 76 °C, which have been shown by X-ray diffraction measurements to correspond to L_β/L_α, L_α/inverted cubic and inverted cubic/inverted hexagonal phase transitions respectively, where L_α and L_β are the lamellar liquid crystalline phase and the lamellar gel phase respectively (D. A. Mannock, unpublished work).

Purified placental glucocerebrosidase was generously provided by Dr. John Barranger, The Children's Hospital, Pittsburgh, PA, U.S.A., and was prepared as described elsewhere [10], and the specific activity of this preparation was 33.3 units/mg of protein. One unit of activity is defined as the amount of enzyme that releases 1 μmol of glucose from MUGlc/min.

Preparation of spleen and brain glucocerebrosidase

Control spleen and brain tissues were obtained at autopsy from individuals free of lipid storage disease; Gaucher spleen and brain tissues were obtained at the time of splenectomy or autopsy. Tissues were stored at −43 °C. Glucocerebrosidase was solubilized with sodium cholate and partially purified as described elsewhere [11].

Standard β-glucosidase assay

The assay medium contained 5 mM-MUGlc, 0.1 M-sodium acetate buffer, pH 5.5, 0.2% (w/v) TDC and 0.1–1.0 μg of placental glucocerebrosidase or 15–150 μg of brain or spleen protein in a final volume of 0.1 ml. Incubations were for 15–60 min at 37 °C. The assays were then terminated by the addition of 2.9 ml of 0.3 M-NH_4OH/glycine buffer, pH 10.5. Fluorescence was determined using a Turner fluorimeter (Model III) and activities were calculated from a standard curve constructed using 4-methylumbelliferone [12]. One unit of β-glucosidase activity is defined as the amount of enzyme that cleaves 1 μmol of glucose from glucoside substrate/min. For the inhibition experiments, various amounts of 2,3-di-14:0-β-Glc-DAG, 0.5 mM-CBE or 1.0 mM-glucosylsphingosine were included in the standard assay medium.

Hexokinase/glucose-6-phosphate dehydrogenase coupled assay

This coupled assay was used to measure the amount of glucose released by hydrolysis of glucocerebroside and 2,3-di-14:0-β-Glc-DAG by different glucocerebrosidase preparations. Placental glucocerebrosidase (0.1–1.0 μg) or spleen or brain glucocerebrosidase preparations (38–380 μg) were incubated with appropriate amounts of 2,3-di-14:0-β-Glc-DAG or glucocerebroside in an assay medium containing 0.1 M-sodium acetate buffer, pH 5.5, and 0.2% (w/v) TDC in a final volume of 0.1 ml. Standard curves were constructed using 0–10 nmol of D-glucose and were included in each set of assays. Incubations were carried out for 90–120 min at 37 °C and terminated by boiling for 2 min. Samples were removed and analysed for glucose by hexokinase phosphorylation of the released glucose in the presence of ATP and then coupled to subsequent $NADP^+$ reduction by glucose-6-phosphate dehydrogenase, which was monitored fluorimetrically as described elsewhere [13,14].

Kinetic analyses

The initial velocities and the corresponding substrate concentrations were subjected to computer analysis according to the method of Cleland [15], which fits the data to a rectangular hyperbola, yielding K_m and $V_{max.}$ parameters. K_i values were determined by replotting of the slope of each line generated by Lineweaver–Burk analysis versus its inhibitor concentration.

Protein determination

Protein was estimated by the method of Lowry *et al.* [16], using BSA as standard.

RESULTS

2,3-Di-*O*-alkyl-1-*O*-(β-D-glycosyl)-*sn*-glycerols as inhibitors of glucocerebrosidase

The presence of a β-D-glucosyl linkage in addition to the two tetradecyl chains in 2,3-di-14:0-β-Glc-DAG (Fig. 1) prompted us to test this compound as an inhibitor of glucocerebrosidase acting on MUGlc. The enzyme is an integral membrane-bound protein *in vivo*; it has a near-absolute dependence on the inclusion of acidic lipids (e.g. phosphatidylserine) or bile salts (e.g. TDC) in assays that measure its activity *in vitro*. As shown in Fig. 2, 2,3,-di-14:0-β-Glc-DAG inhibited glucocerebrosidase when enzyme activity was measured using the standard β-glucosidase assay; in the presence of 0.2% (w/v) TDC, 50% inhibition of MUGlc hydrolysis required approx. 0.15 mM of this glycolipid. The inhibition observed with 2,3-di-14:0-β-Glc-DAG was highly specific, since replacing the glucose moiety of 2,3-di-14:0-β-Glc-DAG with a galactose residue completely abolished inhibition. Noteworthy is the fact that similar concentrations (0.10–0.60 mM) of 2,3-di-14:0-β-Glc-DAG had no effect on the ability of the broad-specificity cytosolic β-glucosidase (from guinea pig liver) to catalyse the hydrolysis of MUGlc at its pH optimum, namely pH 6.0. Likewise, the almond β-glucosidase was not inhibited by 2,3-di-14:0-β-Glc-DAG under the same assay conditions (results not shown). Furthermore, 2,3-di-*O*-tetradecyl-*sn*-glycerol, which is the lipid product generated when glucocerebrosidase acts on 2,3-di-14:0-β-Glc-DAG, had no significant effect on the β-glucosidase activity of placental glucocerebrosidase.

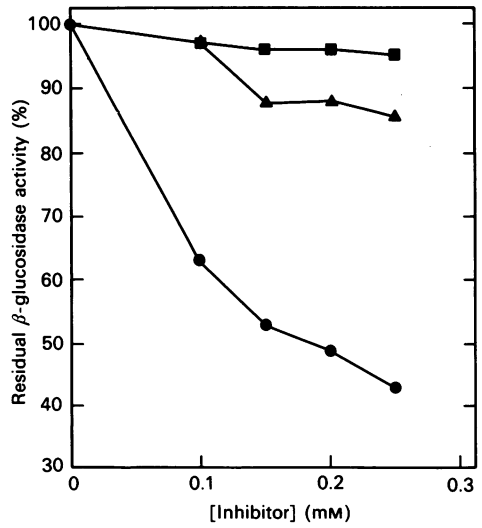

Fig. 2. Inhibition of placental glucocerebrosidase-catalysed MUGlc hydrolysis

Activity was measured in the presence of 0.2% TDC (w/v) plus 2,3-di-O-tetradecyl-sn-glycerol (▲), 2,3-di-14:0-β-Glc-DAG (●) or 2,3-di-14:0-β-Gal-DAG (■).

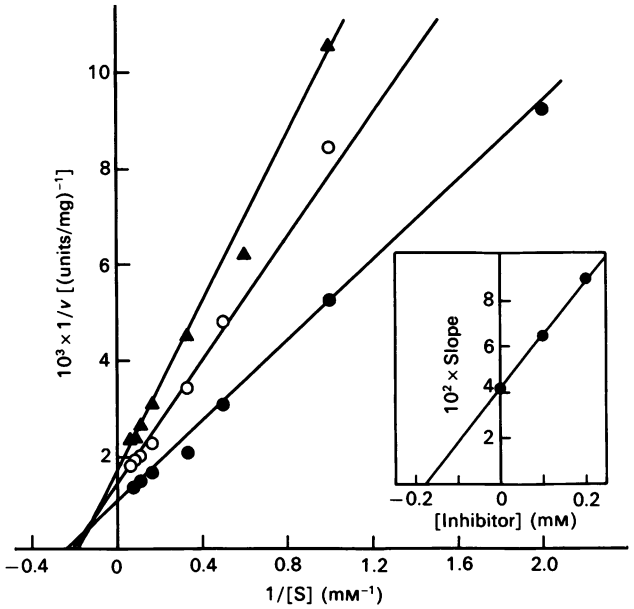

Fig. 3. Lineweaver–Burk analysis of glucocerebrosidase activity

Activity was measured in the absence (●) or the presence of 0.1 mM- (○) or 0.2 mM- (▲) 2,3-di-14:0-β-Glc-DAG using 0.2% (w/v) TDC as the activator. Glucocerebrosidase activity was measured using MUGlc as the substrate in the concentration range 0.5–12 mM in the absence of inhibitor and 1–15 mM in the presence of inhibitor. All data were computer-analysed by the method of Cleland [15] as described in the Materials and methods section. The inset is a replot of the slopes of the Lineweaver–Burk plots versus the respective inhibitor concentration ($r = 0.999$).

Lineweaver–Burk analyses of MUGlc hydrolysis were performed in the absence and presence of 2,3-di-14:0-β-Glc-DAG. As shown in Fig. 3, mixed inhibition was observed and the slope versus inhibitor concentration replot was linear and yielded

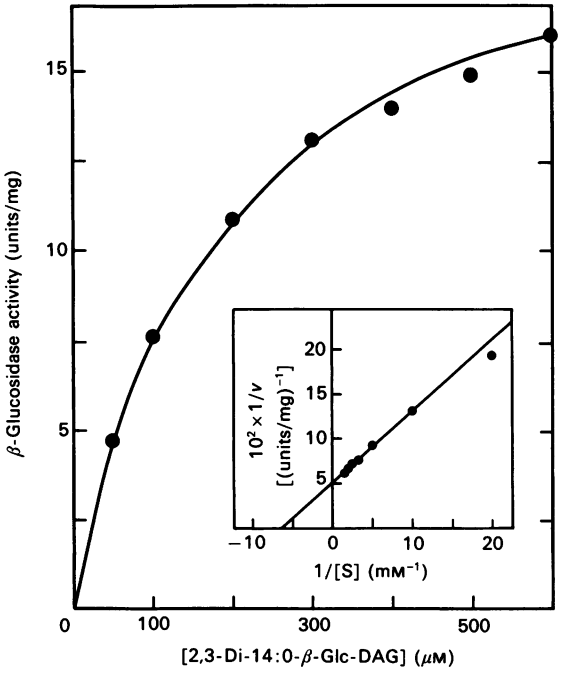

Fig. 4. Placental glucocerebrosidase activity using 2,3-di-14:0-β-Glc-DAG as the substrate in the presence of 0.2% (w/v) TDC

Glucocerebrosidase activity was measured by the hexokinase/glucose-6-phosphate dehydrogenase coupled assay, as described in the Materials and methods section. The inset represents the corresponding Lineweaver–Burk plot of the saturation curve.

an inhibition constant (K_i) of 0.18 mM. These data are consistent with two interpretations, as follows. (i) The inhibitor could interact with the active site and increase the K_m; also, it is conceivable that in the light of its lipoidal character, 2,3-di-14:0-β-Glc-DAG may decrease the effective concentration of TDC in the assay medium by forming mixed micelles with the bile salt and thus decrease the $V_{max.}$. (ii) Alternatively, the inhibitor could bind to the lipid activator (i.e. TDC) site, thus displacing or preventing the binding of TDC to the enzyme and diminishing the $V_{max.}$; furthermore, when bound to this site on the enzyme, the inhibitor could adversely affect the enzyme's affinity for MUGlc and thus contribute a competitive component (i.e. changes in K_m) to the character of the inhibition.

We also performed Lineweaver–Burk analyses with 2,3-di-O-tetradecyl-sn-glycerol as the inhibitor and MUGlc as the substrate; neither K_m nor $V_{max.}$ were altered, even at an inhibitor concentration as high as 0.25 mM.

2,3-Di-14:0-β-Glc-DAG as a substrate for placental glucocerebrosidase

The inhibition observed using 2,3-di-14:0-β-Glc-DAG as the inhibitor and MUGlc as the substrate, taken together with the structural similarities (Fig. 1) between 2,3-di-14:0-β-Glc-DAG and glucocerebroside, caused us to postulate that the former might be serving as a substrate for glucocerebrosidase. Therefore the extent of hydrolysis of 2,3-di-14:0-β-Glc-DAG by placental glucocerebrosidase in the presence of 0.2% (w/v) TDC was measured by estimating the glucose released using a hexokinase/glucose 6-phosphate dehydrogenase coupled assay system. Fig. 4 depicts the change in initial velocity (v) with various 2,3-di-14:0-β-Glc-DAG concentration(s). A Lineweaver–Burk plot of the data (inset to Fig. 4) yielded a K_m

Table 1. Summary of kinetic constants of placental glucocerebrosidase

Values are means ± S.E.M.

Substrate	K_m (mM)	$V_{max.}$ (units/mg)	$V_{max.}/K_m$
MUGlc	4.17 ± 0.30	91.7 ± 0.03	21.8 ± 1.1
Glucocerebroside	0.08 ± 0.01	10.4 ± 0.3	130 ± 10
2,3-Di-14:0-β-Glc-DAG	0.15 ± 0.01	19.8 ± 0.5	132 ± 7

Table 2. Inhibition by CBE and glucosylsphingosine of MUGlc and 2,3-di-14:0-β-Glc-DAG hydrolysis by human placental glucocerebrosidase

The numbers in parentheses denote percentage inhibition of β-glucosidase activity.

	β-Glucosidase activity (units/mg)	
Inhibitor	MUGlc	2,3-Di-14:0-β-Glc-DAG
None	51.8	16.8
CBE (0.5 mM)	10.8 (80)	1.1 (93)
Glucosylsphingosine (1.0 mM)	5.2 (90)	5.0 (70)

Table 3. Substrate competition: comparison of calculated and observed velocities

NA, not applicable.

	Calculated velocity (units/mg)			
Substrate	One enzyme	Two enzymes	Observed velocity (units/mg)	Number of enzymes
[^{3}H]Glucocerebroside (1.1 mM)	9.7	NA	9.5 ± 0.5	NA
[^{3}H]Glucocerebroside (1.1 mM) + 2,3-di-14:0-β-Glc-DAG (0.6 mM)	7.6	9.7	7.3 ± 0.6	1

of 0.15 mM, a value which is close to the apparent K_i value of 0.18 mM which was obtained from the inhibition studies described above (Fig. 3). The kinetic constants (K_m and $V_{max.}$) for MUGlc, glucocerebroside and 2,3-di-14:0-β-Glc-DAG are summarized in Table 1. From these data, 2,3-di-14:0-β-Glc-DAG compares favourably as a substrate for human glucocerebrosidase, expressing a catalytic efficiency ratio ($V_{max.}/K_m$) equal to that of glucocerebroside.

Inhibition of 2,3-di-14:0-β-Glc-DAG hydrolysis by CBE and glucosylsphingosine

Utilizing CBE, a well-documented irreversible inhibitor of glucocerebrosidase, and glucosylsphingosine, a reversible inhibitor of the enzyme [1], we set out to determine if the same enzyme was responsible for the hydrolysis of both MUGlc and 2,3-di-14:0-β-Glc-DAG. The data summarized in Table 2 show that inclusion of 0.5 mM-CBE in the enzyme assays produced an

Table 4. Comparison of β-glucosidase activity in control and Gaucher spleen extracts using MUGlc, 2,3-di-14:0-β-Glc-DAG and glucocerebroside as substrates

For Gaucher patients crude preparations of glucocerebrosidase labelled 1, 2 and 3 were derived from spleens of patients with the adult, non-neurological type 1 form, whereas specimen 4 was from a patient with type 2 neurological Gaucher's disease. ND, not detectable.

	β-Glucosidase activity (munits/mg)		
Source of spleen	Glucocerebroside	MUGlc	2,3-Di-14:0-β-Glc-DAG
Controls			
1	0.34	1.70	0.80
2	0.19	1.87	0.70
3	0.20	1.11	0.40
Mean ± S.D.	0.24 ± 0.08	1.56 ± 0.40	0.63 ± 0.21
Gaucher patients			
1 (type 1)	ND	0.049	0.020
2 (type 1)	ND	0.049	0.022
3 (type 1)	ND	0.029	0.018
4 (type 2)	ND	0.063	0.061

80% loss of MUGlc β-glucosidase activity and a 93% decrease of 2,3-di-14:0-β-Glc-DAG hydrolysis. Likewise, the addition of 1.0 mM-glucosylsphingosine to the assay medium caused 90% and 70% inhibition of MUGlc and 2,3-di-14:0-β-Glc-DAG β-glucosidase activities respectively.

Kinetic competition study

We next performed a kinetic competition study as an alternative approach to test the hypothesis that the same catalytic centre in glucocerebrosidase catalyses the hydrolysis of glucocerebroside and 2,3-di-14:0-β-Glc-DAG. When β-glucosidase activity is measured in the presence of two competing substrates, the hydrolysis of each substrate in the mixture will be significantly less than the hydrolysis of each of them measured individually [14,17,18]. The following equation can be used to calculate the velocity (v) of the reaction of an enzyme that catalyses the hydrolysis of two substrates in a mixture:

$$v = \frac{\frac{V_a[a]}{K_a} + \frac{V_b[b]}{K_b}}{1 + \frac{[a]}{K_a} + \frac{[b]}{K_b}}$$

where V_a and V_b are the maximum velocities, K_a and K_b are the Michaelis constants and [a] and [b] are the concentrations of the two substrates. If two enzymes or two different active sites on the same enzyme are catalysing the hydrolysis of the respective substrates, then the reaction velocity estimated by the release of the common product will be equal to the sum total of the velocities determined with each substrate alone. Alternatively, if hydrolysis of one of the radiolabelled substrates is measured by the release of a radioactive product and there is a decrease in radioactivity as a result of mixing of the radioactive and non-radioactive competing substrates, this will provide evidence for one active site catalysing the hydrolysis of both substrates. We therefore measured the amount of [^{3}H]glucose released when glucocerebrosidase was incubated with [^{3}H]glucocerebroside in the absence and presence of 2,3-di-14:0-β-Glc-DAG. Using the K_m and $V_{max.}$ values derived from the experiments in which

Table 5. Comparison of β-glucosidase activity in control and Gaucher brain extracts using MUGlc, 2,3-di-14:0-β-Glc-DAG and glucocerebroside as substrates

For Gaucher patients, crude preparations of glucocerebrosidase labelled 1 and 2 were derived from brain autopsy samples of a patient who had the type 1 form of the disease and another who had been classified as having the severe infantile neuronopathic Gaucher's disease (type 2).

Source of brain	β-Glucosidase activity (munits/mg)		
	Glucocerebroside	MUGlc	2,3-Di-14: 0-β-Glc-DAG
Controls			
1	0.99	4.25	2.08
2	0.98	5.01	2.47
3	0.44	2.40	1.15
Mean ± S.D.	0.80 ± 0.32	3.89 ± 1.34	1.90 ± 0.68
Gaucher patients			
1 (type 1)	0.09	0.12	0.18
2 (type 2)	0.07	0.08	0.08

[^{3}H]glucocerebroside and 2,3-di-14:0-β-Glc-DAG were compared as placental glucocerebrosidase substrates (Table 1), 1.1 mM-[^{3}H]glucocerebroside in the absence and presence of 0.6 mM-2,3-di-14:0-β-Glc-DAG would be theoretically expected to yield 9.7 and 7.6 μmol/min per mg of glucose respectively if a common catalytic centre was hydrolysing both of these substrates. We observed β-glucosidase activities of 9.5 ± 0.5 and 7.3 ± 0.6 μmol/min per mg in the absence and presence respectively of 2,3-di-14:0-β-Glc-DAG (Table 3), thus validating the contention that the two glycolipids compete for the same catalytic centre on glucocerebrosidase.

Comparison of the rates of hydrolysis of 2,3-di-14:0-β-Glc-DAG by spleen and brain extracts from controls and patients with Gaucher's disease

If the catalytic site in glucocerebrosidase that hydrolyses glucocerebroside is also responsible for the hydrolysis of 2,3-di-14:0-β-Glc-DAG, then patients with Gaucher's disease, whose glucocerebrosidase activity is markedly deficient, should also be compromised in their ability to hydrolyse 2,3-di-14:0-β-Glc-DAG. Hence we compared the abilities of partially purified preparations of glucocerebrosidase from controls and patients with Gaucher's disease to hydrolyse this glycolipid.

Control spleen and brain glucocerebrosidases are capable of hydrolysing 2,3-di-14:0-β-Glc-DAG as well as MUGlc and glucocerebroside (Tables 4 and 5). Splenic enzyme preparations derived from controls hydrolysed 2,3-di-14:0-β-Glc-DAG at approx. 40% of the rate at which they hydrolysed MUGlc (0.63 munit/mg versus 1.56 munits/mg), and more efficiently than hydrolysis of the natural substrate, glucocerebroside (0.24 munit/mg) (Table 4). Control brain extracts exhibited higher activities towards all three substrates compared with control splenic glucocerebrosidases; however, we observed almost the same relative rates of hydrolysis with the three different substrates (Table 5). Crude preparations of control brain glucocerebrosidase hydrolysed 2,3-di-14:0-β-Glc-DAG at 49% of the rate at which it catalysed the hydrolysis of MUGlc (1.9 munits/mg versus 3.9 munits/mg), and at a significantly higher rate than for hydrolysis of glucocerebroside (0.80 munit/mg). In contrast, the Gaucher spleen and brain glucocerebrosidase preparations (from non-neurological and neurological types) were markedly deficient in their abilities to hydrolyse not only glucocerebroside and MUGlc, but also 2,3-di-14:0-β-Glc-DAG (Tables 4 and 5).

Computer modelling of glucocerebroside and 2,3-di-14:0-β-Glc-DAG

In order to gain insight into the structural similarities between glucocerebroside and 2,3-di-14:0-β-Glc-DAG, and to identify the spatial relationship of various functional groups in these two glucocerebrosidase substrates, we undertook a computer-assisted molecular modelling analysis. Using the Alchemy II (Tripos Associates Inc.,) minimizer program we obtained energy-minimized structures of these substrates and assigned either three or four heteroatoms for the superimposition of these two structures. The rationale for choosing (i) the anomeric oxygen of glucose, (ii), C-4 equatorial hydroxy group in glucose and (iii) the ring oxygen in glucose, is accommodated in the following observations. In the two different mechanisms that have been proposed [19] for β-glucosidase action, the first step in each case involves the protonation of the anomeric oxygen before expulsion of the aglycone moiety. Glucocerebrosidase is highly specific for β-D-glucosyl derivatives [20–22] and is incapable of catalysing the hydrolysis of β-D-galactosyl substrates; hence the equatorial hydroxy group at the C-4 atom of glucose must be a critical recognition or attachment site. Substitutions at C-5 of the glucopyranose ring have pronounced effects on the inhibition constants for the interaction of glucose derivatives (e.g. 5-deoxy-5-thio-D-glucose or 1-deoxynojirimycin) with glucocerebrosidase [21]. Comparison of these three co-ordinates in glucocerebroside and 2,3-di-14:0-β-Glc-DAG yielded a good correlation with a root-mean-square deviation of 0.21 Å (0.021 nm) (results not shown). Kinetic studies [20,21] have implicated the presence of a hydrophilic domain in the active site of glucocerebrosidase which interacts with the hydroxy and the amine groups of the sphingosyl

Table 6. Distances between the heteroatoms in glucocerebroside and the corresponding heteroatoms in 2,3-di-14:0-β-Glc-DAG

Energy minimization was performed using the Alchemy II Minimizer software (1 kcal = 4.18 kJ). Dihedral angles are defined by the following atoms: glucocerebroside, O-1–C-1–C-2–N-2; 2,3-di-14:0-β-Glc-DAG, O-1–C-1–C-2–O-2. Distances are those between the oxygen atoms attached to the indicated carbon atoms (also see Fig. 1), with the exception of a nitrogen atom attached to C-2 in glucocerebroside. Root-mean-square (r.m.s.) deviation in Å (1 Å = 0.1 nm) was calculated by Alchemy II for the superimposition of the oxygen atoms attached to C-1, C-2, C-4* and C-5* in 2,3-di-14:0-β-Glc-DAG with the corresponding heteroatoms in glucocerebroside.

Structure	Energy (kcal/mol)	Dihedral angle (°)	Distance (Å)				R.m.s. deviation
			C-1–C-4*	C-1–C-2	C-2–C-4*	C-2–C-5*	
Glucocerebroside	−4.7	139.5	5.67	3.41	6.58	5.07	0.31
2,3,-Di-14:0-β-Glc-DAG	−4.5	102.9	5.69	3.69	7.35	5.50	–

moiety of glucosylceramide. To the above three heteroatoms we added the nitrogen atom in glucocerebroside and the oxygen atom of the C-2 ether linkage of 2,3-di-14:0-β-Glc-DAG, and obtained a four-atom fit with a root-mean-square deviation of 0.31 Å (0.031 nm) (Table 6). The superimposition of the two molecules using the latter four heteroatoms revealed that the hydrophobic (aglycone) and hydrophilic (glycone) moieties in glucocerebroside (−19.6 kJ/mol; −4.7 kcal/mol) displayed a perfect overlap over the corresponding domains in 2,3-di-14:0-β-Glc-DAG (−18.8 kJ/mol; −4.5 kcal/mol) (results not shown). The differences in bond distances between these heteroatoms in the two molecules were minimal, ranging from 0.02 Å to 0.77 Å (0.002–0.077 nm) (Table 6).

DISCUSSION

The major finding in the present study is the demonstration that 2,3-di-14:0-β-Glc-DAG is an excellent substrate for human glucocerebrosidase. The first clue that 2,3-di-14:0-β-Glc-DAG might be a substrate for glucocerebrosidase was the observation that this glycolipid, but not its galactose counterpart (i.e. 2,3-di-14:0-β-Gal-DAG), inhibited the MUGlc/β-glucosidase activity of glucocerebrosidase (Fig. 2). This observation is in agreement with other reports [20–22] in which it has been demonstrated that glucocerebrosidase is highly specific for β-D-glucosides. When the assay medium was supplemented with TDC to activate glucocerebrosidase and disperse lipid substrates, the extensively purified placental enzyme hydrolysed 2,3-di-14:0-β-Glc-DAG as efficiently as it hydrolysed its natural substrate, glucocerebroside (Table 1). The contention that it is glucocerebrosidase which is responsible for the release of glucose from 2,3-di-14:0-β-Glc-DAG is supported by the following observations. (i) Hydrolysis of the latter by extensively purified placental glucocerebrosidase was markedly inhibited by CBE and glucosylsphingosine, both well-established active-site-directed inhibitors of glucocerebroside:β-glucosidase activity. (ii) Glucocerebrosidases extracted from Gaucher spleen and brain tissues were deficient in their ability to hydrolyse 2,3-di-14:0-β-Glc-DAG as well as MUGlc and glucocerebroside.

Taken together with the above findings, the results of the kinetic competition study (Table 3) support the hypothesis that not only do the two β-glucosidase activities reside in the same enzyme molecule, but they also compete for the same active site. Assuming a relatively high degree of specificity for the active site of glucocerebrosidase, we speculated that 2,3-di-14:0-β-Glc-DAG and glucocerebroside molecules have very similar conformations, particularly with regard to those functional groups which interact with amino acid side chains in the enzyme's catalytic centre. We verified this postulate with the aid of computer-assisted molecular modelling. Superimposition of energy-minimized structures of glucocerebroside and 2,3-di-14:0-β-Glc-DAG using four heteroatom co-ordinates resulted in a root-mean-square deviation of 0.31 Å (0.031 nm) (Table 6) and revealed the spatial coincidence of their hydrophilic and hydrophobic constituents (results not shown).

In an effort to further our understanding of the interaction of the enzyme with the substrate, we found it instructive to consider the three-domain model of the active site of glucocerebrosidase that was proposed by Osiecki-Newman *et al.* [21] on the basis of the extensive kinetic study they performed with glucocerebrosidase and several reversible competitive inhibitors and alternative substrates. The model consists of a hydrophilic glycone and two hydrophobic aglycone binding sites, to accommodate the various moieties of the physiological substrate. It is conceivable that the NH- and the OH- groups attached to the C-2 and C-3 atoms in glucocerebroside interact non-covalently with the active site (via hydrogen bonding) and assist in anchoring the two hydrophobic tails in the molecule to the active site. The absence of the type of hydrogen bonding, proposed above, between 2,3-di-14:0-β-Glc-DAG and glucocerebrosidase could account for the higher K_m for 2,3-di-14:0-β-Glc-DAG relative to glucocerebroside.

Although the accumulation of glucocerebroside in the tissues of Gaucher's disease patients is well documented, to our knowledge accumulation of 2,3-di-14:0-β-Glc-DAG or of any other glucoglycerolipid in these patients has never been reported. One could account for this observation on the basis of the absence of any glucoglycerolipids, including 2,3-di-14:0-β-Glc-DAG, in mammalian tissues; however, it is conceivable that enzymes capable of cleaving the acyl/alkyl linkages in these glucoglycerolipids are present in human tissues and could degrade these compounds to glucosylglycerol, which could then be readily excreted.

This pronounced lack of 2,3-di-14:0-β-Glc-DAG hydrolysis by the defective lysosomal β-glucosidase from patients with Gaucher's disease forms a very interesting parallel between Gaucher's disease and Krabbe's disease, another lysosomal storage disease in which the deficient enzyme is galactocerebroside:β-galactosidase. It has been shown that this β-galactosidasehydrolyses1,2-di-*O*-acyl-3-*O*-(β-D-galactopyranosyl)-*sn*-glycerol (1,2-β-Gal-DG) in addition to galactocerebroside, its other natural substrate [23]. More striking is the observation that a β-galactocerebrosidase obtained from extracts of Krabbe tissues is markedly deficient in its ability to hydrolyse 1,2-β-Gal-DG [23], just as the mutant β-glucocerebrosidases from patients with various forms of Gaucher's disease are incapable of hydrolysing 2,3-di-14:0-β-Glc-DAG.

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