N-Hexyl-*O*-glucosyl sphingosine, an inhibitor of glucosyl ceramide β-glucosidase

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Abstract A synthetic analog of glucocerebroside, *N*-hexyl-*O*-glucosyl sphingosine, was found to inhibit the glucosidase in rat spleen that hydrolyzes glucocerebroside. At a concentration of 1 μM, the analog inhibited the enzyme by 48%. The mode of action appeared to be competitive, probably aided by tight binding of the amine group to a carboxyl group near the enzyme's active site. Increasing or decreasing the chain length of the *n*-alkyl group attached to the nitrogen atom led to decreased effectiveness. The inhibitory effect was maximal at pH 7.0, but it was still considerable at the enzyme's optimal pH, 5.0. It is suggested that the compound may be useful for inducing an animal model of Gaucher's disease.

Supplementary key words cerebroside glucosidase · glucosyl sphingosine preparation · cerebroside synthesis

Glucocerebrosidase, the hydrolase that splits the β-linkage in glucosyl ceramide, is a widely distributed, active enzyme (1–4). It is markedly deficient in the human genetic disorder, Gaucher's disease (4–8), and the particle-bound enzyme is greatly stimulated by a glycoprotein found in the cytosol (4).[2] Bile salts, however, also act in the incubation medium to stimulate the enzyme (3). The substrate itself, glucocerebroside, is a minor component of tissues, and its primary metabolic role is probably in the formation (9) and degradation (10) of gangliosides, globosides, and related glycolipids. Thus, the accumulation of glucocerebroside that is characteristic of Gaucher's disease is probably only one of the damaging causative factors in this many-faceted disorder.

The study of Gaucher's disease is severely hampered by the unavailability of an animal model with the same genetic defect. It is possible that a potent synthetic inhibitor which specifically blocks the enzyme in rats would induce a very similar disorder and allow one to follow the details of the developmental changes that ensue when the enzyme is blocked. The availability of such rats would allow one to test inhibitors of ceramide: glucosyltransferase as potential alleviators in Gaucher's disease.

We chose as the starting point in our search for an inhibitor Gatt's observation that sphingosine is a fairly effective inhibitor of the ox brain glucosidase (3). It is not likely that this simple precursor of the sphingolipids could be specific enough to be useful, so we examined derivatives of glucosyl sphingosine. Alkylation of the nitrogen atom with various *n*-alkyl groups yielded effective inhibitors.

MATERIALS AND METHODS

Preparation of labeled substrate

The substrate was made by acylating glucosyl sphingosine with [1-^{14}C]stearic acid. The former compound was made by alkaline hydrolysis from glucocerebroside, which was obtained from a patient with Gaucher's disease who had undergone splenectomy to relieve the discomfort from the enlarged organ. The glucocerebroside was isolated by conventional steps: extraction with C–M 2:1 and washing, alkaline methanolysis of the ester lipids, and Florisil chromatography (11). The yield from 750 g of spleen was 19 g.

The glucosyl sphingosine was made by a slight modifi-

Abbreviations: HGlcS, *N*-hexyl-*O*-glucosyl sphingosine; TLC, thin-layer chromatography; C and M, chloroform and methanol in mixtures (v/v). Glucocerebroside is used as a synonym for the more precise chemical term, glucosyl ceramide.

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[2] The glycoprotein requirement, initially observed with the aid of methylumbelliferyl glucoside, has been demonstrated also with glucocerebroside as substrate (M. W. Ho, J. S. O'Brien, J. S. Erickson, and N. S. Radin, unpublished work).

cation of the method of Taketomi and Yamakawa (12). We refluxed 1.5 g of glucocerebroside for 4 hr with 45 ml of *n*-butanol, 5 ml of water, and 3.5 g of KOH and then dialyzed the mixture for 8 hr against water. (It is important to fill the dialysis tubing slackly, squeezing out the air, as much water enters the sac.) The dialysis was done on a gyratory shaker against frequent water changes with the sacs kept horizontal to make sure the butanol did not float to one end. By this approach we were able to remove the butanol, as well as the KOH, and lyophilize the sac contents without a preliminary vacuum evaporation. We then warmed the powder with 25 ml of C–M 2:1, filtered off insoluble material, and lyophilized the filtrate with the aid of benzene. The fatty acids, residual cerebroside, and some sphingosine were removed by chromatography on a column of Unisil silica gel (200–325 mesh, 40 g/g of lipid, Clarkson Chemical Co., Williamsport, Pa.) packed in C–M 90:10. The lipid was added as a 2% solution in the same solvent, and additional solvent (25 ml/g of Unisil) was then added. The glucosyl sphingosine was eluted with C–M 80:20 or 85:15. Elution was carried out with a column pump and fraction collector, and pure fractions (located by TLC) were pooled and lyophilized. The yield was generally about 500 mg (57% of theoretical).

Acylation with [^{14}C]stearic acid was carried out by a modification of the method of Shapiro and Flowers (13, 14). The labeled fatty acid (93 mg, 4 mCi, from New England Nuclear Corp.) was refluxed 30 min with 4.5 ml of thionyl chloride, cooled in ice, and evaporated to dryness under vacuum. To the reflux vessel were added 223 mg (48% excess) of glucosyl sphingosine in 8 ml of tetrahydrofuran (freshly distilled from KOH) and 6 ml of sodium acetate in water (50%, w/v). The mixture was shaken 30 min and purified by partitioning with 37 ml of C–M 2:1. The lower layer was washed with methanol–water 1:1, evaporated to dryness, and chromatographed on a column of Unisil silica gel, 10 g in a 7.7 × 500 mm column, packed in C–M 97:3. Unreacted stearic acid came out in the first fractions, and glucocerebroside came out in the latter fractions and with C–M 92:8. Unreacted glucosyl sphingosine came out with C–M 85:15.

The yield of cerebroside in trial runs was about 64% of theoretical. Infrared spectra showed the typical peaks obtained with cerebrosides, particularly the split hydroxyl peak at 3 μm and the split *trans* peak (from sphingosine) at 10.1 and 10.4 μm that are characteristic of glucocerebrosides (15). A radioautograph of a TLC plate made with the various radioactive column fractions revealed slight impurities at the origin and just ahead of the cerebroside spot. These amounted to about 0.9% of the total activity. A more highly contaminated column fraction was not used in this study. The labeled product was diluted with nonradioactive stearoyl glucosyl sphingosine made similarly but with equimolar amounts of starting materials. The calculated molecular weight is 728.

Preparation of glucosidase

Spleens (9.6 g) from 10 discarded breeding rats were homogenized in 50 ml of 0.32 M sucrose, first with a Virtis blender, then with a Teflon–glass homogenizer. After a brief centrifugation at 500 *g*, the homogenate was centrifuged for 30 min at 17,000 *g*. The resultant pellet was suspended in more sucrose, final volume 80 ml, yielding a protein concentration (measured with biuret after perchloric acid precipitation) of 1.28 mg/ml. The fine suspension was frozen in 2-ml portions for later use.

The 500–17,000 *g* particles were chosen as the enzyme source as the result of a preliminary survey of spleen glucosidase localization. Additional activity was present in the more difficultly sedimented particles, and a minor amount of activity was found in the cytosol. This is a typical distribution pattern for a lysosomal enzyme. Although the initial description of this hydrolase in rat spleen (2) stated that the activity was predominantly in the cytosol, subsequent observations have shown it to be particulate. It is possible that this error arose from the failure to add bile salt or the cytosol "P factor" (4) to the particles that were assayed.

Other materials

D-Sphingosine and ceramide (stearoyl sphingosine) were prepared from natural materials as described before (16). DL-Dihydrosphingosine was obtained from Miles Laboratories, Kankakee, Ill. Psychosine (galactosyl sphingosine) was made from brain galactocerebroside by the procedure described above. The taurocholate used in the incubations was a purified product from Calbiochem.

Assay method for glucocerebrosidase

The substrate emulsion was prepared by evaporating to dryness a mixture of 2 mg of [^{14}C]stearoyl glucosyl sphingosine (880,000 cpm), 20 mg of Tween 20 (polyoxyethylene sorbitan monolaurate), and 20 mg of Myrj 52 (polyoxyethylene stearate, Atlas Chemicals Division of ICI America). The residue was converted to a clear dispersion with 4 ml of 1% sodium taurocholate solution by sonication in an ultrasonic cleaning bath and brief warming to 70°C.

Each assay tube, a 13 × 100 mm screw-capped test tube, contained 0.4 ml of the substrate, 0.05 ml of 1 M sodium citrate buffer, pH 5, and 0.05 ml of the enzyme preparation, added in that order. The mixture was shaken at 37°C for 2 hr, then processed to isolate the enzymatically produced ceramide. This was done by adding 4 ml of C–M 2:1 containing 0.1 mg of carrier ceramides.

The resultant mixture was centrifuged 30 min, the upper phase was removed, and the surface of the lower phase was washed with 2 × 1 ml of methanol–water 1:1. The material in the lower phase, after drying with nitrogen, was dissolved in 3 ml of C–M 98:2 and applied to a column 4 mm in diameter containing 0.5 g of Unisil, packed in hexane. After an additional 6-ml rinse with C–M 98:2, the combined effluents were evaporated to dryness and counted by liquid scintillation in toluene–alcohol 95:5. Unhydrolyzed cerebroside was recovered from the column by elution with 8 ml of C–M 92:8.

Incubation without enzyme yielded a blank activity of about 160 cpm. A typical incubation yielded about 37 nmoles of ceramide, representing a specific activity of 0.3 μmole/mg of protein/hr and a conversion of about 14% of the substrate. The observed enzyme activity was proportional to enzyme amount, up to at least 0.075 ml of the suspension, but it slowed down slightly during the incubation.

Synthesis of *N*-hexyl-*O*-glucosyl sphingosine

A mixture of 120 mg (0.26 mmole) of glucosyl sphingosine, 0.2 ml of *n*-butanol, and 0.020 ml (0.13 mmole) of 1-bromohexane was heated in a small test tube at 100°C for 1 hr. The dry-looking mixture became a clear liquid on warming and turned somewhat yellow. The tube contents were taken up in 3 ml of 0.07 M methanolic NaOH, 8 ml of chloroform, and 2 ml of water; the mixture was then centrifuged. The upper layer was washed with 8 ml of pure lower phase, the latter was pooled with the first lower layer, and the solution was evaporated to dryness with nitrogen. Analysis of this material by TLC (C–M–water–ammonium hydroxide 70:30:4:1) showed unreacted glucosyl sphingosine at R_F 0.31, a major spot (HGlcS) at R_F 0.56, and a faint spot at 0.68, presumably *N*,*N*-dihexyl glucosyl sphingosine. The two lower spots reacted with ninhydrin, and all three spots gave the blue color typical of amines with alkaline bromothymol blue.

The mixture was separated into its components on a column 5.6 mm in diameter containing 6 g of Unisil in C–M 98:2. The sample was applied to the column in 10 ml of the 98:2 solvent, followed by 50 ml of the same mixture, 120 ml of C–M 95:5, 60 ml of 94:6 (which elutes the dihexyl glucosyl sphingosine), 60 ml of 92:8 (which elutes the monohexyl glucosyl sphingosine), 90 ml of 88:12, and 120 ml of 80:20 (which elutes excess glucosyl sphingosine). Fractions of 30 ml each were collected at a pumping rate of 120 ml/hr. The yield of HGlcS, a clear glass, was 21.5 mg, 30% of theoretical. Elemental analysis of HGlcS by Spang Microanalytical Laboratory, Ann Arbor, Mich., yielded 65.94% C and 10.81% H (theoretical: 66.02% and 10.90%). The calculated molecular weight is 545.8. Further evidence for the structure of HGlcS is that the same compound (compared by TLC color reactions and enzymatic activity) was obtained by reduction of *N*-hexanoyl glucosyl sphingosine.[3] Acid hydrolysis of HGlcS yielded a ninhydrin-positive material migrating ahead of sphingosine on TLC, presumably *N*-hexyl sphingosine.

Other short-chain alkyl glucosyl sphingosines were prepared similarly. While most of the amines came off the column with C–M 97:3, the *N*-butyl derivative required 10% methanol for elution, and the *N*-propyl amine required 12%. In some cases, the TLC spot was characterized additionally with 1-naphthol and sulfuric acid, which gave a positive color for hexose (17). The R_F values of the alkyl glucosyl sphingosines relative to that of glucosyl sphingosine ranged between 1.93 and 2.38, increasing with increasing chain length. The R_F of octadecyl glucosyl sphingosine relative to octadecanoyl glucosyl sphingosine was 1.09, a difference to be expected since amines (in basic solvents) are a trifle less polar than amides.

In the case of octadecyl glucosyl sphingosine, the starting material was the synthetic stearoyl glucocerebroside, which was reduced with Red-al (sodium bis[2-methoxyethoxy]aluminum hydride in benzene, Aldrich Chemical Co.). Cerebroside (20.5 mg) was dissolved in 4 ml of tetrahydrofuran, the reflux vessel was flushed with nitrogen, 1 ml of Red-al was added, and the solution was refluxed for 3.5 hr under a $CaCl_2$ tube. The reactants were treated carefully with 1 ml of water and then 1 ml of 8% NaOH; the reaction mixture was then reduced somewhat in volume with a stream of nitrogen. To the mixture were added 14 ml of C–M 2:1 and 3 ml of water, after which the upper layer (including most of the aluminum hydroxide) was removed. The lower layer, after filtration and evaporation to dryness, showed the secondary amine on TLC, together with some unreduced cerebroside and a fast-moving spot, possibly stearyl alcohol formed by reductive cleavage (18). In agreement with this identification was the finding of an amine with the same R_F as glucosyl sphingosine.

The mixture was purified on a silica gel column as above, but with slight differences in elution steps to improve the separation of the amine from the amide. The final product showed traces of cerebroside as a contaminant.

Each inhibitor was added to the incubation tube as a solution in C–M 2:1, evaporated to dryness with nitrogen, then sonicated with the substrate emulsion and citrate buffer to bring it into solution. The enzyme was then added and incubation was carried out as above. The reported values are the averages of duplicate incubations.

[3] Radin, N. S., and J. S. Erickson. Details to be published later.

TABLE 1. Degree of inhibition of rat spleen glucocerebrosidase by various substances

Compound Tested	Inhibitor Concentration	Extent of Inhibition
	μM	%
D-Sphingosine	300	59
DL-Dihydrosphingosine	300	16
Ceramide	300	5
D-Gluconolactone	300	50
Glucosyl sphingosine	300	75
Galactosyl sphingosine (psychosine)	300	1
N-Octadecyl glucosyl sphingosine	6	56
N-Decyl glucosyl sphingosine	6	74
N-Octyl glucosyl sphingosine	6	79
N-Hexyl glucosyl sphingosine	6	85
N-Butyl glucosyl sphingosine	6	42
N-Propyl glucosyl sphingosine	6	21

Assay conditions as described in the text. The control activity (inhibitor omitted) was about 12,000 cpm, or 37 nmoles of glucocerebroside hydrolyzed in 2 hr.

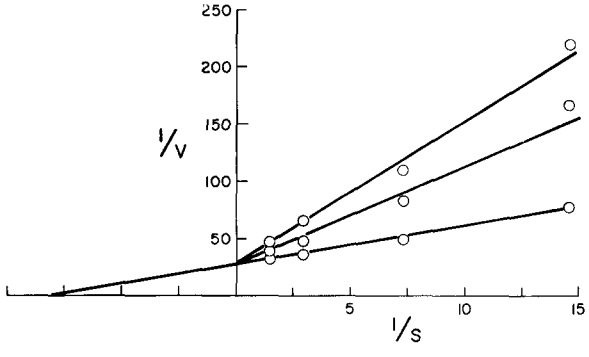

FIG. 1. Effect of hexyl glucosyl sphingosine on glucocerebroside hydrolysis. The bottom line was derived from the control system, with differing concentrations of cerebroside (*S* is in mM), assayed as in the text. The middle line came from incubations in which the inhibitor concentration was 0.4 μM and the upper line came from incubations with 1 μM inhibitor. *V* is expressed in μmoles of ceramide formed in 1 hr. The incubation tubes contained differing amounts of substrate emulsion but equal amounts of taurocholate (4 mg).

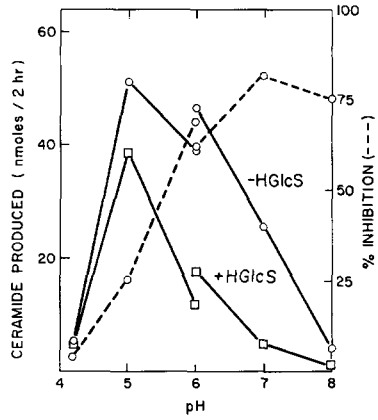

FIG. 2. Effect of incubation pH on enzyme activity and inhibition by *N*-hexyl glucosyl sphingosine (HGlcS). The left side of the drawing was derived from experiments with citrate buffer and the right side from incubations with phosphate buffer. Inhibitor concentration was 0.4 μM.

RESULTS

Comparisons of different inhibitors

Fairly good inhibition was observed with D-sphingosine and gluconolactone, whereas ceramide had virtually no effect (Table 1). These results are in good agreement with those of Gatt (3), who apparently used DL-sphingosine. Unexpectedly, DL-dihydrosphingosine proved to be a poor inhibitor. Glucosyl sphingosine was a little more effective than sphingosine but psychosine had no effect, showing the importance of the glucosyl group. Brady et al. (1) had found that both psychosine and glucosyl sphingosine block glucocerebrosidase almost equally well in tests with a rat intestine preparation, but these were carried out at a very high concentration of inhibitor, which may have raised the pH of the system.

Our first test with *N*-(*n*-decyl)-*O*-glucosyl sphingosine at the 0.3 mM level resulted in complete inhibition of the enzyme, and subsequent evaluations of the alkyl glucosyl sphingosines were made at a lower level (bottom part of Table 1) in order to show the differences in inhibitory effects. The maximal effect was obtained with alkyl chains of 6 to 10 carbon atoms, and the hexyl compound was chosen for further study.

Nature of the inhibition by HGlcS

Tests with different concentrations of HGlcS and substrate showed that HGlcS acts as a competitive inhibitor (Fig. 1). The K_i is about 3.3×10^{-7} M, making this a very effective blocking agent. The K_m for the enzyme in this assay system was 1.25×10^{-4} M, which is comparable to Gatt's (3) value for ox brain of 1.8×10^{-4} M.

Evaluation of the inhibition at different pH's showed that the effect was greatest at pH 7.0 (Fig. 2). Thus, if glucocerebrosidase acts in vivo at a pH corresponding to "typical" intracellular pH, the inhibitory action of HGlcS administered to animals should be quite efficient. Judging by the slightly higher activities observed with the uninhibited enzyme tested with phosphate buffer, it might be better to include phosphate in the citrate buffer we use in the assay system. Ho et al. (5) have shown that citrate + phosphate gave more activity than acetate buffer with human skin fibroblasts.

DISCUSSION

It is increasingly becoming a valid generalization that acid hydrolases possess a somewhat acidic group near the active site. Application of the reasoning of Alberty and

Massey (19) to our pH–activity curve yields a value of roughly 4.5 for the pK_a of an ionizing group near the glucosidase active site (the pH at half-maximal activity on the low side). Such a group, presumably a COOH, could combine firmly with a cationic site in an inhibitor. Evidently, the secondary amine group in HGlcS is strategically located for such a combination. Glucosyl sphingosine coupled to a Sepharose column through an *N*-alkyl chain would probably be useful in the isolation of glucocerebrosidase.

Future attempts at synthesizing an even more effective inhibitor should probably aim at including a second cationic group which could bind to the active site group whose pK_a is indicated indistinctly by the right side of Fig. 2 (roughly 7). A second binding group would also act to ensure greater specificity of the inhibitor.

We have begun a study of the metabolism and metabolic effects of HGlcS, using material labeled in the *n*-hexyl group.

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