Improved Inhibitors of Glucosylceramide Synthase*

(Received for publication, December 28, 1998, and in revised form, March 5, 1999)

Lihsueh Lee, Akira Abe, and James A. Shayman‡

From the Division of Nephrology, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, Michigan 48109

Previous work has led to the identification of inhibitors of glucosylceramide synthase, the enzyme catalyzing the first glycosylation step in the synthesis of glucosylceramide-based glycosphingolipids. These inhibitors have two identified sites of action: the inhibition of glucosylceramide synthase, resulting in the depletion of cellular glycosphingolipids, and the inhibition of 1-O-acylceramide synthase, resulting in the elevation of cell ceramide levels. A new series of glucosylceramide synthase inhibitors based on substitutions in the phenyl ring of a parent compound, 1-phenyl-2-palmitoylamino-3-pyridilino-1-propanol (P4), was made. For substitutions of single functional groups, the potency of these inhibitors in blocking glucosylceramide synthase was primarily dependent upon the hydrophobic and electronic properties of the substituents. An exponential relationship was found between the IC50 of each inhibitor and the sum of derived hydrophobic (π) and electronic (σ) parameters. This relationship demonstrated that substitutions that increased the electron-donating characteristics and decreased the lipophilic characteristics of the homologues enhanced the potency of these compounds in blocking glucosylceramide formation. A novel compound was subsequently designed and observed to be even more active in blocking glucosylceramide formation. This compound, D-threo-4-hydroxy-P4, inhibited glucosylceramide synthase at an IC50 of 90 nM. In addition, a series of dioxane substitutions was designed and tested. These included 3',4'-methylenedioxyphenyl, 3',4'-ethylenedioxyphenyl, and 3',4'-trimethylenedioxyphenyl-substituted homologues. D-threo-3',4'-Ethylenedioxy-P4-inhibited glucosylceramide synthase was comparably active to the p-hydroxy homologue. 4'-Hydroxy-P4 and ethylenedioxy-P4 blocked glucosylceramide synthase activity at concentrations that had little effect on 1-O-acylceramide synthase activity. These novel inhibitors resulted in the inhibition of glycosphingolipid synthesis in cultured cells at concentrations that did not significantly raise intracellular ceramide levels or inhibit cell growth.

GlCer2 is the precursor of hundreds of different glycosphin-

golipids. This cerebroside is synthesized from uridine diphosphate-glucose and ceramide by a glucosyltransferase, GlCer synthase. GlCer-based glycosphingolipids have been identified as important mediators of a variety of cellular functions, including proliferation, differentiation, development, and cell-cell recognition (1). The (R,R)-(d-threo)-isomer of 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and its homologues are potent inhibitors of GlCer synthase. These compounds have been used extensively to study the metabolism and function of glycosphingolipids in living cells (2–6).

In previously reported work, a series of PDMP homologues and analogues was synthesized (4). Replacing the decanoyl moiety with a palmitoyl moiety enhanced the effectiveness of PDMP. In addition, replacing the morpholino ring with a pyrrolidino ring, forming d-threo-1-phenyl-2-palmitoylamino-3-pyridilino-1-propanol (d-threo-P4), also enhanced the inhibitory activity. It was also noted that the d-threo-P4 derivative possessing a p-methoxy substituent on the phenyl group increased the inhibitory activity further. This latter observation led to the present study, an evaluation of other phenyl group substitutions in which the phenyl group of the P4 compound was modified by various electron-donating or -withdrawing groups. As expected, only the d-threo-enantiomers among P4 or P4 derivatives specifically inhibited the enzyme activity. The potency of these compounds in inhibiting GlCer synthase was quantitatively related to the hydrophobic and electronic properties of the phenyl group substitutions of single substituents. This association resulted in the design of a new PDMP homologue (4'-hydroxy-P4) that was significantly more potent than those studied to date.

EXPERIMENTAL PROCEDURES

Materials

The acetoephonones and amines were from Aldrich, Lanca

thome glycosphingosine was prepared as described previously (7).

General Synthesis of Inhibitors

The aromatic inhibitors were synthesized by the Mannich reaction from 2-N-acetylaspartoephene, paraformaldehyde, and pyrrolidine, followed by reduction with sodium borohydride as described previously (2, 4). The reaction produced a mixture of four isomers, due to the presence of two asymmetric carbons. For syntheses in which phenyl-substituted starting materials were used, the chloroacetoephene, methoxyacetoephene, methylenedioxyacetoephene, and methylacetoephene were brominated and converted to the primary amine. Brominations of the methoxyacetoephene, dimethoxyacetoephene, and 3',4'-methylenedioxyacetoephene were performed in chloroform at room temperature, and the products were recrystallized from ethanol acetate and hexane.

The synthesis of 1-(4'-hydroxy)phenyl-2-palmitoylamino-3-pyridilino-1-propanol is described in detail in Fig. 1. This synthesis differed

Received for publication, December 28, 1998, and in revised form, March 5, 1999.

‡ Established Investigator of the American Heart Association. To whom correspondence should be addressed: Nephrology Div., Dept. of Internal Medicine, University of Michigan Medical Center, 1150 West Medical Center Dr., Ann Arbor, MI 48109-0676.

The abbreviations used are: GlcCer, glucosylceramide; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; P4, 1-phenyl-2-palmitoylamino-3-pyridilino-1-propanol; HPLC, high performance liquid chromatography; MDCK, Madin-Darby canine kidney.

This paper is available online at http://www.jbc.org

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.

This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The work was supported by National Institutes of Health Grants R01 DK41487 and RO139255 and by a merit review research award from the Veterans Affairs Medical Center (to J. A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: GlcCer, glucosylceramide; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; P4, 1-phenyl-2-palmitoylamino-3-pyridilino-1-propanol; HPLC, high performance liquid chromatography; MDCK, Madin-Darby canine kidney.

This paper is available online at http://www.jbc.org

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.
Synthesis of

1-(4′-Hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4)

1-(4′-Hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol Formation (Product 2)—4′-Hydroxyacetophenone (compound 1; 13.62 g, 100 mmol), benzyl bromide (17.1 g, 100 mmol), and cesium carbonate (35.85 g, 100 mmol) were added to tetrachloroethane at room temperature and stirred overnight. The product was concentrated to dryness and recrystallized from ether and hexane to yield 15 g of 4′-benzoyloxyacetophenone, which appeared as a white powder. An Rf of 0.42 was observed when resolved by thin-layer chromatography using methylene chloride. 1H NMR (ppm, CDCl3) δ 7.96 (2H, d, 9.8 Hz, O-Ar-C(O)), 7.42 (5H, m, Ar CH(O)-), 7.01 (2H, d, 8.8 Hz, O-Ar-C(O)), 5.14 (2H, s, Ar′ CH(O)-), and 2.56 (3H, s, CH3).

Bromination of 4′-Benzyloxyacetophenone (Product 3)—Bromine (80 mmol) was added dropwise over 5 min to a stirred solution of 4′-benzoyloxyacetophenone (70 mmol) in 40 ml of chloroform. This mixture was stirred for an additional 7 min and quenched with saturated sodium bicarbonate in water until the pH reached 7. The organic layers were combined, dried over MgSO4, and concentrated to dryness. The crude mixture was purified over a silica gel column and eluted with methylene chloride to yield 2-bromo-4′-benzyloxyacetophenone. An Rf of 0.62 was observed when resolved by thin-layer chromatography using ethylene chloride. 1H NMR (ppm, CDCl3) δ 7.97 (2H, d, 9.2 Hz, O-Ar-C(O)), 7.43 (5H, m, Ar′ CH(O)-), 7.04 (2H, d, 9.0 Hz, O-Ar-C(O)), 5.15 (2H, s, Ar′ CH(O)-), and 4.40 (2H, s, CH2Br).

2-Amino-4′-benzyloxyacetophenone HCl Formation (Product 4)—Hexamethylenetetramine (methenamine; 3.8 g, 23 mmol) was added to a stirred solution of 2-amino-4′-benzyloxyacetophenone (6.8 g, 23 mmol) in 100 ml of chloroform. After 4 h, the crystalline adduct was filtered and washed with chloroform. The product was dried and heated with 0.62 g of 10% methanol in dichloromethane. This yielded a mixture consisting of 10% methanol in dichloromethane. This yielded a mixture of 0.42 was observed when resolved by thin-layer chromatography using ethylene chloride. [M + H]+: 479 for C31H45NO3. 1H NMR (ppm, CDCl3) δ 7.96 (2H, d, 8.8 Hz, O-Ar-C(O)), 7.40 (5H, m, Ar′ CH(O)-), 7.03 (2H, d, 8.8 Hz, O-Ar-C(O)), 6.57 (2H, s, NH2), 5.14 (2H, s, Ar′ CH(O)-), 4.71 (2H, s, C(O)CH2NH-C(O)), 2.29 (2H, t, 7.4 Hz, C(O)CH2(CH2)13CH3), 1.67 (2H, m, C(O)CH2(CH2)13CH3), 1.26 (24H, m, C(O)CH2(CH2)13CH3), and 0.87 (3H, t, 6.7 Hz, C(O)CH2(CH2)13CH3).

1′-4′-Benzyloxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol Formation (Products 6 and 7)—2-Palmitoylbenzoyloxyacetophenone (3.79 g, 8.0 mmol), paraformaldehyde (0.25 g, 2.7 mmol, equivalent to 8.1 mmol of formaldehyde), pyridine (0.96 ml, 11.4 mmol), and ethanol (70 ml) were stirred under nitrogen. Concentrated HCl (0.96 ml) was added through the condenser, and the mixture was heated to reflux for 16 h. The resultant brown solution was cooled on ice, and then sodium borohydride (1.3 g, 34 mmol) was added in three portions. The mixture was stirred at room temperature overnight, and the product was dried in a solvent evaporator. The residue was redissolved in dichloromethane (130 ml) and hydrolyzed with 3 N HCl (pH ~4). The aqueous layer was twice extracted with dichloromethane (50 ml). The organic layers were pooled, washed twice with water (30 ml) and twice with saturated sodium chloride (30 ml), and dried over anhydrous magnesium sulfate. The dichloromethane solution was rotary-evaporated to a semisolid and purified by use of a silica rotor using a solvent consisting of 10% methanol in dichloromethane. This yielded a mixture of 0.21 was observed when resolved by thin-layer chromatography using ethylene chloride. [M + H]+: 565 for C36H56N2O3. 1H NMR (ppm, CDCl3) δ 7.96 (2H, d, 9.8 Hz, O-Ar-C(O)), 7.40 (5H, m, Ar′ CH(O)-), 7.03 (2H, d, 8.8 Hz, O-Ar-C(O)), 6.57 (2H, s, NH2), 5.14 (2H, s, Ar′ CH(O)-), 4.71 (2H, s, C(O)CH2NH-C(O)), 2.29 (2H, t, 7.4 Hz, C(O)CH2(CH2)13CH3), 1.67 (2H, m, C(O)CH2(CH2)13CH3), 1.26 (24H, m, C(O)CH2(CH2)13CH3), and 0.87 (3H, t, 6.7 Hz, C(O)CH2(CH2)13CH3).

D-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4)

D-threo-1-[(3′,4′-ethylenedioxy)phenyl]-2-palmitoylamino-3-pyrrolidino-1-propanol

D-threo-1-[(3′,4′-trimethylenedioxy)phenyl]-2-palmitoylamino-3-pyrrolidino-1-propanol

D-threo-1-[(3′,4′-methylenedioxy)phenyl]-2-palmitoylamino-3-pyrrolidino-1-propanol

D-threo-4′-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4)

FIG. 1. Synthetic pathway for 1-(4′-hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol.

from those of the other compounds because of the need for the placement of a protecting group on the free hydroxyl (product 2) and its subsequent removal (product 8). All other syntheses employed a similar synthetic scheme.

FIG. 2. Structures of P4 and the phenyl-substituted P4 homologues used in this study.
The reactions were carried out in the absence or presence of varying concentrations of P4 (B) or p-methoxy-P4 (C). After filtration, the crystallized aminoacetophenone HCl was washed with cold isopropyl alcohol and then with ether. The yield of this product was ~7.1 g (81%).
layer was extracted once with chloroform (50 ml). The organic solutions were pooled and rotoevaporated until almost dry. The residue was redissolved in chloroform (100 ml) and crystallized by the addition of hexane (400 ml). The flask was then cooled to 4 °C for 2 h. The crystals were filtered and washed with cold hexane until they were almost white and then dried in a fume hood overnight. The yield of the product was 27 mmol (11.6 g).

\[ \text{threo-1-3',4',4'-Ethyleneedioxyphenyl-2-palmitoylamino-3-pyrrolidino-1-propanol} \]

The dichloromethane solution was rotoevaporated to a viscous oil, which was purified by use of a Chromatotron with a solvent consisting of 10% dichloromethane, and the borate complex was hydrolyzed with HCl (3N) until the pH was 4. The aqueous layer was extracted twice with 50 ml of dichloromethane. The organic layers were pooled, washed twice with H2O (30 ml) and saturated NaCl (30 ml), and dried over anhydrous MgSO4. The dichloromethane solution was rotoevaporated to a viscous oil, which was purified by use of a Chromatotron with a solvent consisting of 10% methanol in dichloromethane to obtain a mixture of threo- and erythro-enantiomers (2.24 g, 4 mmol).

Resolution of Inhibitor Enantiomers

High performance liquid chromatography (HPLC) resolution of the threo- and erythro-enantiomers was performed using a preparative HPLC column (Chirex 3014, (S)-Val-(R)-1-(o-naphthyl)ethyamine, 20 × 250 mm; Phenomenex Inc.) eluted with hexane/1,2-dichloroethane/ethanol/trifluoroacetic acid (64:30:5.74:0.26) at a flow rate of 8 ml/min. The column eluent was monitored at 254 nm in both the preparative and analytical modes. The isolated products were reinjected until pure by analytical HPLC analysis using an analytical Chirex 3014 column (4.6 × 250 mm) and the same solvent mixture at a flow rate of 1 ml/min.

GlcCer Synthase Activity

The enzyme activity was measured as described previously (8). Madin-Darby canine kidney (MDCK) cell homogenates (120 µg of protein) were incubated with uridine diphosphate-[3H]glucose (100,000 cpm) and liposomes consisting of 85 µg of octanoylsphingosine, 570 µg of dioleoylphosphatidylcholine, and 100 µg of sodium sulfatide in a 200-µl reaction mixture and kept for 1 h at 37 °C. P4 and P4 derivatives dissolved in dimethyl sulfoxide were dispersed into the reaction mixture after adding the liposomes. The final concentration of dimethyl sulfoxide was <1%. At this concentration, there was no inhibition of the enzyme activity.

Cell Culture and Lipid Extraction

MDCK cells (5 × 10^5) were seeded into 10-cm dishes containing 8 ml of serum-free supplemented Dulbecco’s modified Eagle’s medium (9). After 24 h, the medium was replaced with 8 ml of the medium containing 0, 1, 3, 11, or 113 nM threo-P4, threo-3',4'-ethylenedioxy-P4, or threo-4'-hydroxy-P4. The GlcCer synthase inhibitors were added to the medium as a 1:1 molecular complex with delipidated bovine serum albumin (4, 6). The cells were incubated for 24 or 48 h with the inhibitors. After the incubation, the cells were washed twice with 8 ml of cold phosphate-buffered saline and fixed with 2 ml of cold methanol. The fixed cells were scraped and transferred to a glass tube. An additional 1 ml of methanol was used to remove the remaining cells in the dish.

Three ml of chloroform were added to the tube and briefly sonicated using a water bath sonicator. After centrifugation at 800 × g for 5 min, the supernatant was transferred into another glass tube. The residues were reextracted with chloroform/methanol (1:1). After the centrifugation, the resultant supernatant was combined with the first one. The residues were air-evaporated and kept for protein analysis by the bicinchoninic acid method. NaCl (0.9%) was added to the supernatant, and the volume of chloroform/methanol/water was adjusted to 1:1:1. After centrifugation at 2000 × g for 5 min, the upper layer was discarded. The lower layer was washed with methanol/water (1:1) in an volume equal to that of the lower layer. The resultant lower layer was transferred into a glass tube and dried under a stream of nitrogen gas. A portion of the lipid was used for lipid phosphate determination (10). The remainder was analyzed using high performance thin-layer chromatography.

RESULTS

Resolution of PDMP Homologues by Chiral Chromatography

The structures of the parent compound, threo-P4, and the phenyl-substituted homologues including the new dioxy-substituted and 4'-hydroxy-P4 homologues are shown in Fig. 2. Initially, the effect of each P4 isomer separated by chiral chromatography on GlcCer synthase activity was determined (Fig. 3A). Four peaks were observed for the chiral separation of P4. Peaks 1 and 2 represent the erythro-diastereomers, and peaks 3 and 4 represent the threo-diastereomers as determined by a sequential separation of the P4 mixture by reverse-phase chromatography followed by the chiral separation. The enzyme activity was specifically inhibited by the fourth peak, the threo-isomer (Fig. 3B). This specificity for the threo-enantiomer was consistent with the previous results observed in PDMP and PDMP homologues (2–4). The IC50 of threo-P4 was 0.5 µM for GlcCer synthase activity measured in the MDCK cell homogenates.

Effects of P4 and P4 Derivatives with a Single Phenyl Group

FIG. 4. Relationship between the inhibition of GlcCer synthase activity and σ + π. The data were plotted from those shown in Table I. [●] p-chloro-P4; [■] m-methyl-P4; [▲] p-methoxy-P4; [+, P4]; [●] p-methoxy-P4; [○] 4'-hydroxy-P4.

FIG. 5. Effects of threo-4'-hydroxy-P4 compared with threo-p-methoxy-P4 on GlcCer synthase activity.
Substitution on GlcCer Synthase Activity—In previous work, the addition of a p-methoxy group to DL-threo-P4 was found to enhance the effect of the inhibitor on the enzyme activity (4). This improved efficacy was confirmed for the DL-threo-enzyme. The transacylase activity was potently inhibited by DL-threo-p-methoxy-P4 (IC50 = 0.2 μM) (Fig. 3C). Chiral chromatography of the four p-methoxy-P4 enantiomers failed to resolve completely to base line each enantiomer (Fig. 3A). A slight inhibition of the enzyme activity by p-methoxy-P4 in a combined mixture of DL-erythro- and DL-threo-enantiomers (peaks 2 and 3) was observed; this might have been due to contamination of the DL-threo-isomer (peak 4) into these fractions, although this is not apparent on the separation shown.

Next, a series of DL-threo-P4 derivatives containing other single substituents on the phenyl group was investigated. The potency of these phenyl-substituted compounds as GlcCer synthase inhibitors was inferior to that of DL-threo-P4 or p-methoxy-DL-threo-P4 (Table I). For many drugs, the effect of aromatic substitutions on the biological activity has been characterized mainly dependent upon two factors (hydrophobic and electronic properties) of a substituent of the phenyl group (Table I). The IC50 values (data not shown).

The hydrophobic effect (π) is described by the equation 
\[ \log \frac{F_X}{F_H} = \alpha \log K_X + \beta \log K_H + \gamma \]  
where \( F_X \) and \( F_H \) are the partition coefficients of the substituted and unsubstituted compounds, respectively. The electronic substituent parameter (σ) was originally developed by Hammett (12) and is expressed as 
\[ \sigma = -\log K_X - \log K_H \]  
where \( K_X \) and \( K_H \) are the ionization constants for a para- or meta-substituted aromatic compound.

The p-Hydroxy-substituted Homologue Is a Significantly Better GlcCer Synthase Inhibitor—The strong association between π + σ and GlcCer synthase inhibition suggested that a still more potent inhibitor could be produced by increasing the electron-donating properties and decreasing the lipophilic properties of the phenyl group substituent. A predictably negative π + σ value would be observed for the p-hydroxy homologue. This compound was synthesized (see “Experimental Procedures”), and the DL-threo-enantiomer was isolated by chiral chromatography. An IC50 of 90 nM for GlcCer synthase inhibition was observed (Fig. 5), suggesting that the p-hydroxy homologue was twice as active as the p-methoxy compound. Moreover, the linear relationship between \( \log(1/IC_{50}) \) and π + σ was preserved (Fig. 4).

Effects of DL-threo-3',4'-Dioxy-P4 Derivatives on GlcCer Synthase Activity—To further evaluate the potential effects of structurally similar but more complex substitutions, new DL-threo-P4 derivatives with methylenedioxy, ethylenedioxy, and trimethylenedioxy substitutions on the phenyl group were designed (Fig. 2). The enzyme activity was most strongly inhibited by DL-threo-3',4'-ethylenedioxy-P4, with an IC50 value of 100 nM (Fig. 6). On the other hand, the IC50 values for DL-threo-3',4'-methylenedioxy-P4 and DL-threo-3',4'-trimethylenedioxy-P4 were ~500 and 600 nM, respectively.

Interestingly, DL-threo-3',4'-dimethoxy-P4 was inferior to these dioxy derivatives, even to DL-threo-P4 or m- or DL-threo-p-
Glucosylceramide Synthase Inhibition

Table II
Glucosylceramide, and diradylglycerol content of MDCK cells treated with D-threo-P4, D-threo-ethylenedioxy-P4, and D-threo-4-hydroxy-P4

| Condition | Ceramide | Diradylglycerol |
|-----------|----------|----------------|
|           | pmol/nmol phospholipid | pmol/nmol phospholipid |
| Control   | 4.53 ± 0.12 | 24.2 ± 2.36 |
| 24 h      | 6.88 ± 0.49 | 32.3 ± 3.11 |
| 48 h      | 8.07 ± 0.59 | 39.8 ± 1.11 |
| D-threo-P4 |          |               |
| 11.3 nm   | 5.33 ± 0.41 | 24.1 ± 1.66 |
| 24 h      | 5.68 ± 0.27 | 29.6 ± 0.73 |
| 48 h      | 7.08 ± 0.29 | 33.0 ± 2.63 |
| 113 nm    | 4.64 ± 0.38 | 26.6 ± 1.56 |
| 24 h      | 7.08 ± 0.29 | 33.0 ± 2.63 |
| 48 h      | 9.74 ± 0.53 | 38.8 ± 1.11 |
| D-threo-4′-Hydroxy-P4 | | |
| 11.3 nm   | 4.29 ± 0.71 | 30.9 ± 2.01 |
| 24 h      | 6.70 ± 0.29 | 38.4 ± 1.44 |
| 48 h      | 7.47 ± 0.29 | 41.5 ± 0.66 |
| 113 nm    | 5.09 ± 0.95 | 31.5 ± 3.84 |
| 24 h      | 7.47 ± 0.29 | 41.5 ± 0.66 |
| 48 h      | 9.74 ± 0.53 | 38.8 ± 1.11 |
| D-threo-3′-Ethylenedioxy-P4 | | |
| 11.3 nm   | 5.24 | 22.0 |
| 24 h      | 5.04 | 24.7 |
| 48 h      | 5.21 | 32.5 |
| 113 nm    | 9.64 | 32.5 |
| 24 h      | 13.0 | 41.6 |

a p < 0.05 by Student’s t test.

1.49 as previously described (9). For D-threo-P4, D-threo-3′,4′-ethylenedioxy-P4, and D-threo-4′-hydroxy-P4 for GlcCer synthase were studied by assaying the transacylase. Although there was a significant difference in activity among D-threo-3′,4′-ethylenedioxy-P4, D-threo-4′-hydroxy-P4, and D-threo-P4 in inhibiting GlcCer synthase, the D-threo-enantiomers of all three compounds demonstrated comparable activity in blocking 1-O-acylceramide synthase (Fig. 7).

To determine whether inhibition of 1-O-acylceramide synthase was the basis for inhibitor-mediated ceramide accumulation, the ceramide and diradylglycerol levels of MDCK cells treated with D-threo-P4, D-threo-3′,4′-ethylenedioxy-P4, and D-threo-4′-hydroxy-P4 were measured (Table II). Significant increases in both ceramide and diradylglycerol occurred only in cells treated with inhibitor concentrations in excess of 1 μM. This was ~30-fold lower than the concentration required for inhibition of the 1-O-acylceramide synthase assayed in the cellular homogenates. This disparity in concentration effects most likely reflects the ability of the more potent homologues to accumulate within intact cells (6).

Effects of D-threo-P4, D-threo-4′-Hydroxy-P4, and D-threo-3′-Ethylenedioxy-P4 on GlcCer Synthesis and Cell Growth—To confirm the cellular specificity of D-threo-3′,4′-ethylenedioxy-P4 and D-threo-4′-hydroxy-P4 as compared with D-threo-P4, MDCK cells were treated with different concentrations of the inhibitors. Approximately 66% and 78% of the GlcCer was lost from the cells treated with 11.3 nM D-threo-4′-hydroxy-P4 and D-threo-3′-ethylenedioxy-P4, respectively (Fig. 8, B and C). By contrast, only 27% depletion of GlcCer occurred in cells exposed to D-threo-P4 (Fig. 8A). A low level of GlcCer persisted in the cells treated with a 113 or 1130 nM concentration of either compound. This may be due to the contribution, by degradation, of more highly glycosylated sphingolipids or the existence of another GlcCer synthase that is insensitive to the inhibitor.

On the other hand, there was little difference in the total protein content between untreated cells and those treated with 11.3 or 113 nM D-threo-4′-hydroxy-P4 and D-threo-3′-ethylenedioxy-P4 (Fig. 8, B and C). A significant decrease in total protein was observed in the cells treated with a 1130 nM concentration of either P4 homologue. In addition, the level of ceramide in the cells treated with 1130 nM D-threo-3′-ethylenedioxy-P4 and D-threo-4′-hydroxy-P4 was two times higher than that measured in the untreated cells (Table II). There was no change in ceramide or diradylglycerol levels in cells treated with 11.3 and 113 nm concentrations of either compound. Similar patterns for GlcCer levels and protein content were observed after 48-h incubations (data not shown).

The phospholipid content was unaffected at the lower concentrations of either D-threo-ethylenedioxy-P4 or D-threo-4′-hydroxy-P4. The ratios of cell protein to cellular phospholipid phosphate (μg of protein/nmol of phosphate) were 4.94 ± 0.30, 5.05 ± 0.21, 4.84 ± 0.90, and 3.97 ± 0.29 for 0, 11.3, 113, and 1130 nM D-threo-ethylenedioxy-P4, respectively, and 4.52 ± 0.30, 4.35 ± 0.10, and 3.68 ± 0.99 for 11.3, 113, and 1130 nM D-threo-4′-hydroxy-P4, respectively, suggesting that the changes in GlcCer content were truly related to inhibition of GlcCer synthase activity. These results strongly indicate that two new inhibitors, D-threo-4′-hydroxy-P4 and D-threo-3′,4′-ethylenedioxy-P4, are able to potently and specifically inhibit GlcCer synthesis in intact cells at low nm concentrations without any inhibition of cell growth.

methoxy-P4, as an inhibitor (Fig. 6). As the parameters ơ_m and ơ_p, (the Hammett constants for the meta- and para-substitutions, respectively) and π for a single methoxy substituent are 0.12, −0.27, and −0.02, respectively (11), the value of π + ơ for D-threo-dimethoxy-P4 is presumed to be negative. Therefore, based only on the electronic substituent parameters, D-threo-dimethoxy-P4 deviates quite far from the correlation observed in Fig. 4. This may be due to a repulsion between two methoxy groups in the dimethoxy-P4 molecule that induces a steric effect that was negligible in mono-substituted D-threo-P4 derivatives studied in Fig. 4. GlcCer synthase is thought to possess a domain that interacts with D-threo-PDMP and PDMP homologues and that modulates the enzyme activity (2, 6). The steric effect generated by an additional methoxy group may affect one or more of these interaction domains. As a result, the potency of the dimethoxy homologue as a GlcCer synthase inhibitor was markedly decreased.

Distinguishing between Inhibition of GlcCer Synthase and 1-O-Acylceramide Synthase—Prior studies on PDMP and related homologues revealed that both the threo- and erythro-diastereomers were capable of increasing cell ceramide and inhibiting cell growth despite the observation that only the D-threo-enantiomers blocked GlcCer synthase (4). An alternative pathway for ceramide metabolism was subsequently identified (the acylation of ceramide at the 1-hydroxyl position) that was blocked by both threo- and erythro-diastereomers of PDMP. The specificities of D-threo-P4, D-threo-3′,4′-ethylenedioxy-P4, and D-threo-4′-hydroxy-P4 for GlcCer synthase were studied by assaying the transacylase. Although there was a significant difference in activity among D-threo-3′,4′-ethylenedioxy-P4, D-threo-4′-hydroxy-P4, and D-threo-P4 in inhibiting GlcCer synthase, the D-threo-enantiomers of all three compounds demonstrated comparable activity in blocking 1-O-acylceramide synthase (Fig. 7).

Prior studies on PDMP and re-...
DISCUSSION

Since the original description of an inhibitor of GlcCer synthesis by Vunnam and Radin (13), the pharmacological blockade of glycosphingolipid synthesis has proven to be a valuable approach to understanding the metabolism and function of glycosphingolipids. Previous work with the parent GlcCer synthase inhibitor (PDMP) identified two concurrent effects in cells. These included the time-dependent depletion of all GlcCer-based glycosphingolipids and the accumulation of ceramide. Originally, the ceramide accumulation was believed to be the result of substrate accumulation. However, it was discovered that the erythro-enantiomers of pyrrolidino-substituted compounds raised cell ceramide independent of GlcCer depletion (4). In addition, homologues with aliphatic substitutions depleted GlcCer with minimal effects on ceramide levels. The current data with the new phenyl-substituted inhibitors are consistent with this observation. No growth inhibition was observed at low nM concentrations of either D-threo-ethylenedioxy-P4 or D-threo-4′-hydroxy-P4, but was observed at higher concentrations at which ceramide levels increase. These findings suggested a second site of action for these inhibitors that is independent of the inhibition of GlcCer synthase.

The search for another site of inhibition of ceramide metabolism in the presence of either threo- or erythro-diastereomers of P4 led to the identification of a novel pathway for ceramide metabolism, the acylation of ceramide at the 1-hydroxyl position. The formation of this lipid is catalyzed by a novel phospholipase A2. In the presence of ceramide as an acceptor, 1-O-acylceramide synthase can transacylate ceramide utilizing the sn-2-fatty acid of phosphatidylethanolamine or phosphatidylcholine (14). This transacylase has recently been purified (15). By using the erythro-diastereomers of inhibitors that increase ceramide to the exclusion of blocking GlcCer formation, it has been determined that the growth inhibitory effects of these homologues are mediated by ceramide accumulation and not GlcCer depletion.

The dissociation of GlcCer depletion from ceramide accumulation is an important and necessary finding if one is to con-

Fig. 8. Effects of P4 and related homologues on GlcCer synthesis and cell growth. MDCK cells were treated for 24 h with or without different concentrations of D-threo-P4 (A), D-threo-4′-hydroxy-P4 (B), and D-threo-ethylenedioxy-P4 (C). The total protein amount in each sample was determined by the bicinchoninic acid method (dotted lines). In the GlcCer analysis, lipid samples and standard lipids were applied to the same high performance TLC plate pretreated with borate and developed in a solvent consisting of chloroform/methanol/water (63:24:4). The level of GlcCer was estimated from a standard curve obtained using a computerized image scanner (solid lines). The values were normalized on the basis of the phospholipid content. Each point shown represents the average values from three dishes, with error bars corresponding to 1 S.D.
sider the development of GlcCer synthase inhibitors as potential drugs for inherited glycosphingolipid storage diseases. Ideally, such a drug should exhibit little or no cellular toxicity. The growth inhibitory and proapoptotic effects of ceramide should ideally be eliminated. Recently, support for the concept of treating sphingolipid storage disorders by inhibition of GlcCer synthase was reported by the reversal of the Tay-Sachs phenotype in knockout mice treated with a structurally unrelated inhibitor of GlcCer synthesis, N-butyldeoxynojirimycin (16). This inhibitor is significantly less potent and less specific than the compounds characterized in the present report.

Previous refinements of the parent structure of PDMP have resulted in compounds with greater activity against GlcCer synthase. However, these substitutions of the fatty acyl chain and cyclic amine moieties were designed empirically. In the present study, single phenyl substitutions permitted the analysis of inhibitor activity based on constants derived by Hansch and others many years ago (18). Parameters of both lipophilicity and electronegativity were observed to be predictive of inhibitory activity. Specifically, the design and synthesis of D-threo-4'-hydroxy-P4 yielded the most potent GlcCer synthase inhibitor to date. These parameters, however, could be applied only to simple substitutions and were inadequate to explain the structure activity profile of more complex ring structures. Nevertheless, the further application of these principles may result in newer glycolipid synthase inhibitors with even greater activity and specificity.

Acknowledgment—We gratefully acknowledge the role of Norman Radin in reviewing this manuscript.

REFERENCES
1. Hakomori, S., and Igarashi, Y. (1993) Adv. Lipid Res. 23, 147–162
2. Inokuchi, J., and Radin, N. S. (1987) J. Lipid Res. 28, 565–571
3. Abe, A., Inokuchi, J., Jimbo, M., Shimeno, H., Nagamatu, A., Shayman, J. A., Shukla, G. S., and Radin, N. S. (1992) J. Biochem. (Tokyo) 111, 191–196
4. Abe, A., Radin, N. S., Shayman, J. A., Wotring, L. L., Zipkin, R. E., Sivakumar, R., Ruggieri, J. M., Carson, K. G., and Ganem, B. (1995) J. Lipid Res. 36, 611–621
5. Rani, C. S. S., Abe, A., Chang, Y., Rosenwzeig, N., Saltiel, A. R., Radin, S. A., and Shayman, J. A. (1995) J. Biol. Chem. 270, 2859–2867
6. Abe, A., Radin, N. S., and Shayman, J. A. (1996) Biochim. Biophys. Acta 1299, 331–341
7. Abe, A., Wu, D., Shayman, J. A., and Radin, N. S. (1992) Eur. J. Biochem. 210, 765–773
8. Shukla, G., Shukla, A., Inokuchi, J., and Radin, N. S. (1991) Biochim. Biophys. Acta 1083, 101–108
9. Shayman, J. A., Mahdiyoun, S., Deshmukh, G., Barcelona, F., Inokuchi, J., and Radin, N. S. (1990) J. Biol. Chem. 265, 12135–12138
10. Ames, B. N. (1966) Methods Enzymol. 8, 115–118
11. Högberg, T., and Norinder, U. (1991) in A Textbook of Drug Design and Development (Larsen, P. K., and Bundgaard, H., eds) pp. 55–91, Harwood Academic Publishers, Philadelphia
12. Hammett, L. P. (1940) Physical Organic Chemistry, McGraw-Hill Book Co., New York
13. Vunnam, R. R., and Radin, N. S. (1980) Chem. Phys. Lipids 26, 265–278
14. Abe, A., Shayman, J. A., and Radin, N. S. (1996) J. Biol. Chem. 271, 14383–14389
15. Abe, A., and Shayman, J. A. (1998) J. Biol. Chem. 273, 8467–8474
16. Platt, F. M., Noises, G. R., Reinkerzeimer, G., Townsend, M. J., Perry, V. H., Proia, R. L., Winchester, B., Dwek, R. A., and Butters, T. D. (1997) Science 276, 428–431
17. Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E., and Bell, R. M. (1986) J. Biol. Chem. 261, 8597–8600
18. Tute, M. S. (1970) Adv. Drug Res. 6, 1–77