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Use of an Inhibitor of Glucosylceramide Synthesis, D-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol

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Methods for the synthesis, purification, and use of an inhibitor of glucosylceramide synthesis (PDMP or D-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) are given. The inhibitor is effective with a variety of cells and animals in producing a depletion of glucosylceramide. Because this cerebroside is the precursor of hundreds of other glycolipids, depletion of all of these compounds also takes place as each one is degraded by hydrolases. Use of PDMP causes accumulation of ceramide, the lipoidal precursor of glucosylceramide. Some of this simple sphingolipid is diverted to the synthesis of sphingomyelin and some, via hydrolysis, to formation of sphingols (sphingoid bases). The above changes in the biosynthesis of glycolipids result in pronounced effects on cells: slowing of growth, increased activity of the phospholipase C that catalyzes phosphatidylinositol bisphosphate hydrolysis, accumulation of *N,N*-dimethylsphingosine (an inhibitor of protein kinase C), accumulation of diacylglycerol (an activator of PKC), and reduction of the ability to bind to extracellular matrix proteins. PDMP is rapidly absorbed and released by cells. In mice, it is metabolized by a microsomal monooxygenase and the products are excreted. The degradative process can be blocked by inhibitors of cytochrome P-450, such as piperonyl butoxide, cimetidine, and fluconazole. Understanding these properties permits the use of PDMP in both *in vitro* and *in vivo* studies in which glycolipids may exhibit important biological effects. Two recent examples of the *in vitro* and *in vivo* use of PDMP are provided. © 1993 Academic Press, Inc.

Glucosylceramide (GlcCer)[1] is the precursor of hundreds of structurally distinct glycosphingolipids, found in a wide variety of organisms and cell types. To distinguish these lipids from fucosylceramide and galactosylceramide and their anabolites, we use the term glucosphingolipids (GSLs). Specific GSLs have been found to be involved in or required for a variety of physiological phenomena, including cell signaling events, cell–cell binding, binding of cells to matrix proteins, metastasis of tumor cells, binding of viruses, toxins, and microorganisms to cells, cell growth, cell differentiation, and the degree of malignancy of cancer cells. Several novel GSLs have been found only in tumors, while other—normally occurring—GSLs have been found at abnormally high levels in tumors. Several GSLs occur at very high levels in patients with genetic disorders of GSL metabolism, such as Gaucher disease in the case of GlcCer. Each of these disorders ("sphingolipidoses") is characterized by a wide assortment of specific symptoms, hinting at many unexpected roles for these GSLs.

It is possible to deplete cells of their GSLs by blocking GlcCer synthesis, presumably because the higher GSLs present at the time GlcCer synthesis is inhibited all undergo turnover and are hydrolyzed by a variety of specific glycosidases. GlcCer itself also undergoes hydrolysis by a β-glucosidase. Thus, the first effect of synthase inhibition is a decrease in the *de novo* synthesis of GlcCer. The more complex GSLs (which also begin to disappear via hydrolysis) form GlcCer so that the total level of GlcCer is temporarily maintained. No doubt there is some temporary cannibalization of intermediate GSLs for continued synthesis of specific GSLs, but this process too must come to a halt. In an intact organism, as opposed to cultured cells, there may be some redistribution of GSLs between organs via the serum lipoproteins. In a culture medium supplemented with serum, the serum lipoproteins will temporarily furnish GSLs to the cells. There may be redistribution between individual cells or subcellular membranes by action of glycolipid transfer proteins and there is certainly appreciable excretion of GSLs by the kidney. In the case of cultured cells, especially cancer cells, there is secretion of GSLs into the medium. This is called "shedding" by Li and Ladisch (1). Such tumor products find their way into the patient's plasma, where they can be evaluated for diagnosis and monitoring of therapy.

[1] Abbreviations used: GlcCer, glucosylceramide; GSL, glucosphingolipid; hR_f, percentage R_f ($100 \times R_f$); MDCK cells, Madin–Darby canine kidney cells.

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Inhibiting GlcCer synthase causes accumulation of its lipoidal precursor, ceramide, a lipid that may exert its own physiological effects. It has not yet been shown that UDP-Glc, the other precursor of GlcCer synthesis, also accumulates; it is possible that unused nucleotide sugar is converted to UDP-Gal or other nucleotides or that a feedback process slows its synthesis. Ceramide accumulation has been demonstrated in cultured cells but not in intact mice. The consequences of ceramide accumulation are discussed below.

In this article we describe the use of PDMP (D-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) to block the synthesis of GlcCer. The compound (Fig. 1) was originally developed for use in the treatment of Gaucher disease and the higher sphingolipidoses (2). In the case of the former disorder, most patients suffer from a marked but incomplete deficiency in the ability to hydrolyze GlcCer. Thus, GlcCer, formed *de novo* and by breakdown of the higher GSLs, accumulates and produces a variety of pathological symptoms. The accumulation process is typically very slow and it appears likely that the patient has almost enough glucosidase activity to avoid the accumulation process. It seemed possible that the use of a synthase inhibitor might balance the rates of synthesis and hydrolysis and, perhaps, give the stored GlcCer a chance to reenter the anabolic or catabolic pathways.

STRUCTURE OF PDMP

The structure of the active isomer of PDMP has not been firmly established. On the basis of reported structures of two starting materials, it was thought to have the *threo* structure (7); however, it now appears likely that the active isomer has the *erythro* structure, like that of sphingosine. To distinguish between our previous identification and the new one, we indicate the structure in parentheses: (*erythro*). The Chemical Abstracts name is Decanamide, *N*-[2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl] and the Chemical Abstracts Registry numbers for the different isomers are [130981-80-5], [109836-82-0], and [130981-81-6].

PDMP clearly resembles the structures of both ceramide and GlcCer. The phenyl group in PDMP is the analog of the long alkyl chain of sphingosine, even mimicking the bend in the chain due to sphingosine's *trans* double bond. The decanoylamino structure closely mimics the acylamino structure in sphingolipids. The hydroxyl group adjacent to the benzene ring resembles the 3-hydroxyl group of sphingosine. The morpholine ring resembles the pyranosyl ring of glucose. The morpholine ring also resembles the diethylamino group typical of so many useful drugs. The tertiary nitrogen atom in the ring gives PDMP its cationic charge when dissolved in water, improves its solubility in water, and is important for the compound's inhibitory action.

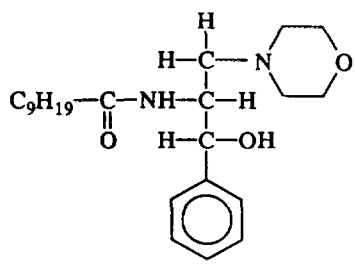

FIG. 1. Structure of D-PDMP.

It is likely that sugar transferases, which bind a nucleotide sugar somewhere in the active site, possess an anionic site (perhaps aspartic or glutamic) to which the sugar moiety is temporarily transferred. Thus, it makes sense to synthesize a substance possessing a cationic group in this region. Some of the compounds synthesized before arriving at PDMP had an amine group nearby in a different structure; these also exhibited some inhibitory activity (2).

Substantial inhibitory activity was obtained with an analog in which the morpholine ring was replaced with piperidine (2). Other secondary amines also showed some activity. However, PDMP hydrochloride proved to be the easiest to purify, so it was used for all further work. (A typographical error in Ref. 2, Table VI, identifies the piperidine compound, RV-471, as the morpholine compound.)

DL-PDMP · HCl and its palmitoyl homolog (PPMP), as well as the resolved enantiomers of PDMP, are currently available from Matreya, Inc. (Pleasant Gap, PA 16823) and Biomol Research Laboratories (Plymouth Meeting, PA 19462). Currently, however, the costs of these inhibitors make their use for *in vivo* work very expensive. The methods for the synthesis and purification of PDMP are therefore provided.

ORGANIC SYNTHESIS OF PDMP

Organic reagents were from Aldrich Chemical Co. and TLC plates (silica gel 60, 0.25 mm thick) were from EM Separations (E. Merck).

The most economical approach for large-scale preparations is to start with acetophenone, brominate it in chloroform solution, and convert it to 2-aminoacetophenone by condensation with hexamethylenetetramine and hydrolysis of the adduct with HCl (3). The hydrolysis step requires some trial and error in the procedure for separating the product (aminoacetophenone · HCl) from contaminating ammonium chloride. It is also possible to buy 2-bromoacetophenone (phenacyl bromide) and 2-aminoacetophenone · HCl (phenacylamine · HCl).

Phenacylamine · HCl (I)

The details for amination of bromoacetophenone are as follows (4). Hexamethylenetetramine (methenamine, 28 g, 0.20 mmol) is added to a stirred solution of phenacyl bromide (39.8 g, 0.20 mmol) in 300 ml of chloroform. After 2 h, the crystalline adduct is filtered and washed with chloroform. It is then dried and refluxed with 190 ml of methanol and 76 ml of concd HCl for 30 min. On cooling, precipitated ammonium chloride is removed by filtration and the filtrate is left in a freezer overnight. The crystallized phenacylamine · HCl, after filtration, is washed with cold isopropanol and then with ether. The yield of **I** is ~19.8 g (61%, MW 171.6, mp 195–196°C, with dec.)

2-Decanoylaminoacetophenone (II)

Ketoamine **I** is acylated with decanoyl chloride in the presence of tetrahydrofuran and 50% (w/v) NaOAc · $3H_2O$ (5, 6). The acetate solution is made from 100 g of NaOAc · $3H_2O$ and 127 ml water. (Although anhydrous NaOAc can also be used, with extra water, it forms hard aggregates that take much stirring to dissolve.) The THF is freshly distilled from KOH pellets to remove peroxides, but one should avoid distilling down to a small volume of THF, especially if it is old and peroxide-ridden.

The reaction is carried out by mixing 100 mmol (21.16 g) of aminoacetophenone · HCl with 150 ml of THF, stirring vigorously with a metal propeller in a 1000-ml beaker. To this is added 100 ml of acetate solution and then 120 mmol (25 ml) of decanoyl chloride (dropwise, over 7–10 min). The reaction is stirred further at high speed for 60 min more, during which period a bulky yellow precipitate forms and needs periodic loosening from the beaker walls.

The amide is recovered by partitioning in a 1000-ml separatory funnel, using 320 ml of chloroform–methanol (2:1) and 20 ml of water. The two layers clear on sufficient standing. The lower layer is washed twice with 100-ml portions of methanol–saline (1:1) and then rotoevaporated to a syrup that promptly crystallizes on cooling. The crystals are dissolved in 25 ml of warm chloroform and reevaporated to a syrup. Now the residue is recrystallized from 200 ml of hot hexane, left in the cold, filtered, and rinsed with cold hexane.

The air-dried product weighs ~24.5 g (85% of theoretical). Thin-layer chromatography (TLC) of a 20-μg band with chloroform–methanol (99:1) yields a single spot at hR_f 44 and a very small trace spot at hR_f 62.

2-Decanoylamino-3-morpholinopropiophenone (III)

Compound **II** is next condensed with formaldehyde and morpholine, forming DL-2-decanoylamino-3-morpholinopropiophenone (7). Compound **II** (20 g, 68 mmol) is refluxed for ~16 h with paraformaldehyde (1.68 g, equivalent to 55.4 mmol of formaldehyde) in 80 ml of 95% ethanol, 8.4 ml of morpholine (95.8 mmol, >99% pure), and 2.5 ml of concd HCl. (The HCl is first mixed with the ethanol, which is then added to the morpholine.) The solution darkens during heating.

TLC with chloroform–methanol–HOAc (90:5:10) shows the ketone band at hR_f 57 but a fast moving band can be seen at hR_f 92.

The melting point of the free base is 79–80°C after recovery by partitioning and crystallization from benzene–hexane. The hydrochloride, crystallized from ethyl acetate, melts at 137–138°C (8). This compound is a relatively weak inhibitor of GlcCer synthase, but it completely inactivates the enzyme *in vitro* and *in vivo* (2), evidently because it reacts covalently with a group near the active site.

DL-threo- and erythro-PDMP (IV)

The DL-morpholino ketone **III** can be reduced to the alcohol without isolating it from the reaction mixture. Sodium borohydride (4.8 g) is dissolved in 25 ml of water containing 50 μl of 50% NaOH and then diluted with 75 ml of absolute ethanol. The cloudy suspension is added slowly to prevent foaming, with mixing, to the ice-cold reaction mixture (formed as above). The mixture is then stirred 3 h more at room temperature, adjusted to pH ~4 (wet pH paper) with 125–150 ml of 1 M HCl, and extracted with 150 ml of chloroform. The upper layer is washed with 100 ml of chloroform and the two lower layers are pooled. The pooled layers are then washed twice with 100 ml of 10% NaCl, dried with Na_2SO_4 to remove the turbidity, and rotoevaporated to an oil. Caution is needed when rotation of the flask is stopped while the vacuum is still present.

The syrup is dissolved in some chloroform and reevaporated to a more viscous syrup, which is then crystallized from 140 ml of chloroform and 280 ml of diethyl ether (freshly distilled from KOH). Crystal formation is sometimes slow at room temperature and is helped by the addition of seed crystals. The mixture is swirled as the crystals form and then is swirled in the cold room overnight (in a flask well sealed to prevent an explosion). Filtration, with rinses of chloroform–ether (1:4) and drying in air yield DL-*threo*- and DL-*erythro*-PDMP · HCl in ~1:1 ratio.

Four isomeric products are formed because two asymmetric centers are present. The *erythro* and *threo* forms are readily separated by TLC with chloroform–methanol–HOAc (90:10:10); hR_f = ~28 and 34. The bands can be detected with methanolic iodine, alkaline bromothymol blue, or a copper sulfate–phosphoric acid charring reagent (9). This last is made from 124 g of $CuSO_4$ hydrate, 44 ml of phosphoric acid, 648 ml of water, and 80 ml of methanol.

Assays of GlcCer synthase showed that the lower PDMP band (eluted with chloroform–methanol) was strongly inhibitory, while the upper band was inactive. It may be noted that *threo*-sphingosine, examined by TLC with a similar solvent and silica gel, migrates a little below the *erythro* form.

ISOLATION OF THE ACTIVE ISOMER OF PDMP

Separation of the threo and erythro Isomers

The crystals obtained from the first crystallization step are stored under vacuum or recrystallized from 75 ml each of chloroform and ether, as before. These crystals are dried under vacuum, to yield ~8.9 g of DL-(*erythro*)-PDMP · HCl. This is ~30% of theoretical based on the weight of 2-decanoylaminoacetophenone. Some analyses indicated the presence of a half molecule of water, making the MW 436 ($C_{23}H_{40}O_{3,5}N_2Cl$), while other analyses, particularly of the D-enantiomer, indicated that it is a monohydrate (MW 445). The melting point of the DL-(*erythro*) isomer is ~155–156°C.

At times, particularly during the early synthetic trials, the above separation of the two forms has failed, even when seed crystals were used. Possibly the amount of water present after the drying step with Na_2SO_4 is a factor. Sometimes the first crop is enriched in the *threo* form, in which case the filtrate generally yields a good preparation of the *erythro* form. Possibly, heavy use of seed crystals or a short crystallization time would help. Most recently, we have been investigating the use of the toluenesulfonic acid salt rather than the HCl salt. In preparing longer chain fatty acyl homologs of PDMP, chloroform–hexane of varying ratios has been used (10).

Efforts at using silica gel column chromatography, which readily separated the *threo* and *erythro* isomers with a chloroform–methanol–HOAc mixture, failed to yield the expected products and it is possible that contact with silica gel or HOAc over a period of time causes some kind of rearrangement reaction. Good separations were also produced, on a much smaller scale, with a C_8-derivatized silica gel HPLC column, using 10 mM dibutylamine phosphate, pH 3, and acetonitrile (60:40). The effluent was monitored at the absorption maximum, 206 nm (7).

Separation of the D- and L-Enantiomers

Resolution of DL-(*erythro*)-PDMP is accomplished (a) by crystallizing the PDMP salt of an optically active acid (7) or, on a smaller scale, (b) by converting the inhibitor to the ester of an optically active acid and separating the isomers chromatographically (11), or (c) by using a chiral column (10).

In the first method, 2.5 g of the hydrochloride is converted to the free base by partitioning between 120 ml of chloroform–methanol (2:1) and 30 ml of 1 M ammonium hydroxide (7). The lower layer is washed with 70 ml of MeOH–saline (1:1) to remove most of the ammonia and ammonium chloride and then with 50 ml of water. The lower layer is rotoevaporated to an oil, dissolved in diethyl ether, dried with Na_2SO_4, and again evaporated to an oil. The yield of free base appears to be quantitative (2.25 g).

Eighty milliliters of acetone and 2.19 g (5.81 mmol) of (+)-dibenzoyl-D-tartaric acid in 80 ml of acetone are added to this oil. The solution is stirred at room temperature for no more than 4 h, as the undesired salt eventually also tends to precipitate. The crystals of monotartrate monohydrate ($C_{23}H_{38}O_3N_2 \cdot C_{18}H_{14}O_8 \cdot H_2O$ = MW 766.9) are washed with 2 × 10 ml of acetone. The yield is ~1.24 g (1.62 mmol = 56% of theoretical). The melting point is 158–159°C.

The filtrate from the crystallization step is evaporated to dryness, the residue is dissolved in 80 ml of warm acetone, and crystallization is allowed to proceed (with seed crystals) by stirring for 5 h. After washing, this yields a second crop weighing 0.40 g, with the same melting point. The salts are converted to the free base with chloroform–methanol–ammonium hydroxide as above, which brings the dibenzoyltartrate into the upper, water-rich phase. This ammonium salt can be recycled for further resolutions. The lower layer of the partition is converted to the desired salt by addition of the appropriate acid (usually HCl) and evaporation to dryness. It is possible that, for some applications, it is not necessary to change the salt form.

Resolution can be carried out on a smaller scale by dissolving DL-(*erythro*)-PDMP · HCl in a 10% solution of (1*R*)-(−)-camphanic acid chloride (2.5 mol eq) in pyridine. Pyridine sold and stored under nitrogen appears to be superior to ordinary pyridine. After 18 h, excess acid chloride is destroyed by adding excess methanol and leaving the mixture for another hour. The pyridine base is removed by rotoevaporation with toluene in a well-sealed evaporator.

The mixture of esters, separated by TLC with chloroform–methanol–HOAc–acetonitrile (92:3:4:2), yields two bands of hR_f 30 and 33. When the esterification was carried out with D-PDMP resolved by the tartrate salt method, only the *upper* band was seen (7). Thus, the camphanate reaction is a good test of enantiomeric purity. A useful feature of the esterification method is that one need not separate the *threo* and *erythro* diastereomers before carrying out the esterification (the inactive isomers migrate below the active ester).

The D-(*erythro*)-camphanate ester can be eluted from a TLC plate with chloroform–methanol–water (7:7:1) and cleaved by methanolysis. Alternatively, it can be isolated by HPLC with a mixed-function column (cation/C_8, Alltech Associates) (11) using the solvent mixture aqueous potassium phosphate, pH 4–acetonitrile–methanol (45:37:20). The ester is readily cleaved by leaving it in chloroform–methanolic NaOH (2:1) for 1 h. The latter solution is nominally 0.21 M NaOH in methanol. The NaOH is removed by partitioning with $\frac{1}{4}$ volumes of isotonic saline and then by washing the lower layer a few times with methanol–saline (1:1) until the upper layer shows only a pale pink color with phenolphthalein. The

resultant free base is converted to the desired salt as above and crystallized once.

A convenient method of resolving the hydrochloride salts on a small scale is the use of a chiral column [cellulose tris(3,5-dimethylphenylcarbamate) on silica gel] using hexane–isopropanol (9:1). Monitoring of the column effluent can be done at 254 nm since the sample load is high in the preparative mode. In the analytical mode, the maximal absorbance peak is at 206 nm with the hydrochloride in isopropanol. Curiously, the specific absorbance and location of the UV maximum depend on the PDMP concentration. The column is available in Japan as Opti-Pak XC (Waters, Millipore) and in the United States as Chiralcel OD (Chiral Technologies, Exton, PA). Although this method was used primarily for analytical evaluation of the resolution process, it was also useful for small-scale preparation of D- and L-[^{3}H]PMMP [the myristoyl or tetradecanoyl homolog (10)]. The D-enantiomers elute before the L-enantiomers. Unfortunately, the chiral column cannot be used without first separating the *threo* and *erythro* racemates.

RELATED COMPOUNDS

Fluorescent "PDMP"

Rosenwald *et al.* have described a fluorescent version of PDMP (12). They removed the decanoyl group of PDMP by heating it with 0.8 M methanolic KOH under nitrogen for 18 h at 100°C and extracted the deacylated compound ("lyso-PDMP") with ether. This was acylated with a fluorescent acyl ester of *N*-hydroxysuccinimide. This sequence of reactions could also be used to attach a novel or radioactive fatty acid. The free base is available from Matreya, Inc.

Radioactive D-(erythro)-PDMP

Radioactive D-(*erythro*)-PDMP has been made by reducing 2-decanoylamino-3-morpholino-propiophenone · HCl with labeled $NaBH_4$ (11). The use of a small proportion of [^{3}H]borohydride yielded preferential reduction to the *erythro* form.

PDMP Homologs

Variants of PDMP differing in acyl chain length were compared with D-(*erythro*)-PDMP for activity against GlcCer synthase (10, 13). Surprisingly, the C_8 to C_{18} compounds did not differ very much in their ability to inhibit the enzyme *in vitro*. The break point appeared with the C_6 homolog, suggesting that the enzyme has two cavities, one that binds an alkyl chain of ~6 methylene groups relatively weakly, and a second, adjacent cavity that binds additional methylene groups quite strongly. Incubating the homologs with MDCK cells for 24 h (10) revealed marked differences in their ability to slow cell growth. For example, at 8 μM, the hexanoyl derivative reduced thymidine conversion to DNA by only 15%, while PDMP reduced it by 42% and the stearoyl derivative blocked it almost completely. The C_{14} and C_{16} homologs were more effective than the C_{18}. Similar differences were seen when cell protein was measured. There was not a simple linear dose–response curve relating GlcCer levels to cell growth.

Some clarification of the difference between homologs was obtained by labeling both PDMP and PMMP with tritium and incubating them at 2 μM with MDCK cells. The uptake of the longer chain inhibitor was 20 times that of PDMP after 30 min and 46 times as high after 24 h. These differences could be due to differences in permeation rate or binding affinity (by lysosomes?). Whatever the explanation, it was demonstrated by TLC that GlcCer synthesis by intact cells was much more sensitive to the myristoyl derivative.

Differences in sensitivity to PDMP homologs were seen also in the ability of the compounds to inhibit the growth of Ehrlich ascites carcinoma cells in mice (14).

The L-Enantiomer of PDMP

L-(*erythro*)-PDMP can be made from DL-(*erythro*)-PDMP by forming the salt of dibenzoyl-L-tartaric acid according to the method using the D-tartaric ester.

In some studies with cultured cells, L-PDMP caused an increase in the level of lactosylceramide and ganglioside GM3 (15). In others, 10 μM L-PDMP produced a marked loss of GSLs, including GlcCer and lactosylceramide (16–18). However, D-PDMP was much more effective. The mechanism of L-PDMP action remains to be discovered. In intact mice, it had no effect on the amounts of ceramide, GlcCer, lactosylceramide, and sphingomyelin in kidney (9).

USING PDMP IN *IN VITRO* STUDIES

PDMP has now been shown to exert a multitude of biological effects in both *in vitro* and *in vivo* studies. Studies utilizing exogenously administered GSLs to assess their function have the potential problem of producing artifactual changes due to the displacement by the added lipids of endogenous compounds. By contrast, PDMP and its homologs have the advantage of altering the metabolism of endogenously formed GSLs.

PDMP · HCl can be used in culture media, without detergent, by evaporating the stock isopropanol solution in a sterile flask with sterile nitrogen, adding sterile medium, and sonicating in a water bath (16). It can also be prepared as a 4 mM solution in water and filter-sterilized (19). However, the longer acyl homologs of PDMP, even though they can be used at a lower concentrations, may need a little detergent or strong sonication with a dipping probe. We have found that the probe can be used under sterile

conditions if a thin sheet of rubber, with a small hole bored in the center, is pushed up the probe so that it is just above the mouth of the medium container. The rubber and lower part of the probe are washed with 70% alcohol and the sonicator is used in a sterile hood. With ethanol-resistant cells, ethanolic inhibitor can be added directly to the medium, which is then sonicated in a bath. Visual observation of the stored dispersion's clarity is advisable.

PDMP · HCl seems to be quite stable in aqueous solution, as evaluated by TLC and radioautography of [^{3}H]PDMP. However, there have been some tentative indications that the solution in isopropanol loses its effectiveness even when stored in a freezer, so more study is needed. The acetate and hydrochloride salts are readily soluble in isopropanol or ethanol, but the latter may be undesirable because of its tendency to form acetaldehyde on storage.

In addition, we have found that aqueous PDMP is adsorbed to some extent by the plastic used in cell incubations (10). Thus, it is probably more active in cells than predicted from its initial concentration in the medium. The possibility that other components of culture media and added substances are adsorbed by the plastic does not seem to have been investigated by other investigators, but it is known that the source of culture dishes can be a significant variable.

Inhibition of GlcCer synthase by PDMP was not time-dependent, suggesting it acted as a competitive inhbitor. When assayed with lyophilized mouse brain as the source of GlcCer synthase, 38 μM PDMP produced 84% inhibition. From kinetic evaluations, it seemed that PDMP is an uncompetitive inhibitor with respect to UDP-glucose and a mixed-type competitive inhibitor against ceramide (7). The measured K_i depends on the nature of the enzyme source and the composition of the assay medium. For enzyme assay methods that simply measure the radioactivity in the organic layer (after incubation with UDP-[^{3}H]glucose and partitioning), it is important to check the radioactive products to make sure that the particular enzyme source being used does not catalyze the synthesis of lipids other than GlcCer.

Investigations *in vitro* with PDMP have been directed primarily toward understanding the manner by which glycosphingolipids may regulate important cellular processes, including growth, hormone signaling, and adhesion. Investigations *in vivo* have been directed toward understanding the potential role of GSLs in the pathophysiology of specific diseases, including cancer and diabetic nephropathy. In the following sections we review the use of PDMP in an *in vitro* study of phospholipase C regulation and an *in vivo* study of streptozotocin-induced diabetic hypertrophy.

The Madin–Darby canine kidney (MDCK) cell line was obtained from the American Type Culture Collection (Rockville, MD). This cell line has a well-characterized hormonal response to bradykinin, resulting in the hydrolysis of phosphatidylinositol bisphosphate with generation of inositol 1,4,5-trisphosphate ($InsP_3$).

The above considerations were applied to the use of PDMP in the study of GSL-mediated phospholipase C activity (20). Addition of either D- or DL-(*erythro*)-PDMP to MDCK cells resulted in a time- and concentration-dependent decrease in glucosylceramide and ganglioside GM3, the major acidic GSL in this cell line. GSL levels can be measured in a relative way by radiolabeling cells with [^{3}H]galactose (5 mCi/ml; sp act, 30 Ci/mmol) or directly by charring and densitometric quantitation. In the former case, GlcCer was radiolabeled because UDP-[^{3}H]galactose is readily converted to UDP-[^{3}H]glucose by a cellular epimerase. After the end of the incubation period, the cells were scraped from their culture dishes and extracted with chloroform–methanol–water (30:60:8). Centrifugation at 15,000*g* sedimented the cell protein. The supernatant was removed for isolation of the GSLs. The neutral and acidic GSLs were separated by DEAE-Sephadex chromatography (acetate form) and the cerebrosides were further separated by silicic acid chromatography as described (21).

The changes in the neutral and acidic GSL levels of MDCK cells are shown in Fig. 2 and 3. Treatment with PDMP resulted in significant decreases in GlcCer and ganglioside GM3 as assessed by both [^{3}H]galactose incorporation and charring.

The potential effects of GSL depletion on hormone-stimulated inositol trisphosphate formation were evaluated in plasma membranes isolated from MDCK cells. Membranes were isolated by exposure of cultured cells to

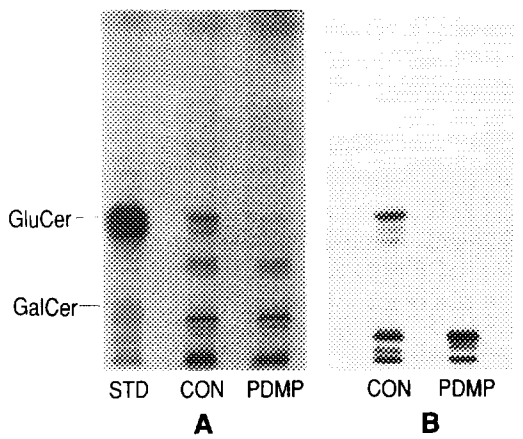

FIG. 2. Effect of PDMP on synthesis of glucosylceramide. MDCK cells were grown in control medium or medium supplemented with 20 μM PDMP for 48 h. During the last 24 h, [^{3}H]galactose was added. The lipids were extracted and the neutral lipids were chromatographed on borate-impregnated HPTLC plates as described. Cerebrosides were detected by charring (A) or by autofluorography (B) and identified by comparison with standards (STD). Glucosylceramide, the major cerebroside of strain I. MDCK cells, was only slightly radiolabeled in the PDMP-treated cells. Reproduced, by permission of the publisher, from Ref. 20.

a hypotonic buffer following the protocol of Hepler and Harden (22). The membranes were isolated by centrifugation at 12,000*g*. The membranes were then resuspended in a buffer consisting of 10 mM Hepes, 2 mM EGTA, 424 μM $CaCl_2$, 0.91 mM $MgSO_4$, 115 mM KCl, and 5 mM KPO_4, pH 7.0. To initiate $InsP_3$ formation, additions of the same buffer were made to the membrane preparations in order to achieve final concentrations of GTP-γS and/or bradykinin of 1.0 and 0.1 μM, respectively. At the end of the incubation period trichloroacetic acid was added to attain a final concentration of 6%. The resultant precipitate was sedimented by centrifugation at 5000*g*. The supernatant was removed and treated with trioctylamine–Freon to extract the acid. $InsP_3$ levels were measured with a competitive binding assay utilizing high-specific-activity *myo*-[2-^{3}H]inositol 1,4,5-trisphosphate and calf adrenocortical microsomes as previously detailed (23). Residual $InsP_3$ 5-phosphatase activity, which varies from preparation to preparation, can be inhibited by the inclusion of 2 mM 2,3-bisphosphoglycerate in the incubation buffer (24). This effectively blocks the degradation of $InsP_3$ and increases the measured levels of $InsP_3$ more than eightfold.

Figure 4 displays the time-dependent effect of PDMP on GlcCer mass and $InsP_3$ formation in MDCK cells. In this study MDCK cells were exposed to 20 μM PDMP for varying periods of time. The plasma membranes were then isolated and stimulated with GTPγS and bradykinin or buffer alone. PDMP treatment resulted in a marked enhancement of stimulated $InsP_3$ formation.

The mechanism for the increase in $InsP_3$ formation following GSL depletion by PDMP is uncertain. Similar changes have been observed following down-regulation of protein kinase C activity by phorbol esters (25). In a subsequent study we observed that there is both a time- and concentration-dependent loss of cytosolic protein kinase C activity in MDCK cells treated with PDMP (26). One plausible explanation for this change in protein kinase C activity is that accumulation of ceramide stimulates the formation of sphingomyelin and diglyceride due to phosphorylcholine transfer from phosphatidylcholine to ceramide. Thus, the change may be due to an accumulation of diacylglycerol. Preliminary studies in support of this pathway have been reported (27).

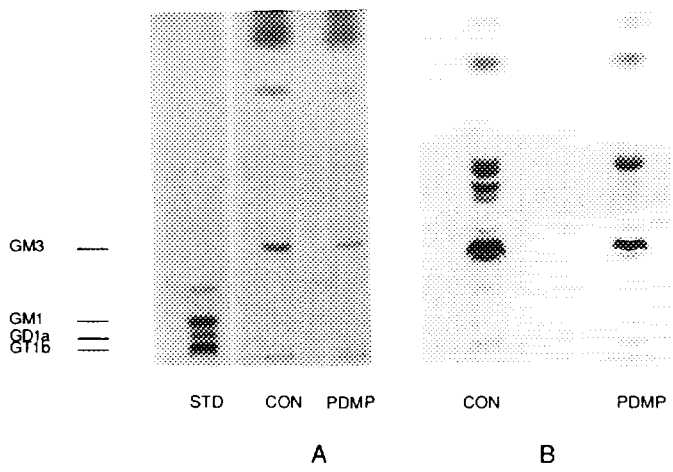

FIG. 3. Effect of PDMP on the acidic glycolipids of MDCK cells. The cells were processed as in Fig. 2 but the acidic lipids were chromatographed. Gangliosides were detected by charring (A) or by autofluorography (B) and identified by comparison with standards (STD). Reproduced, by permission of the publisher, from Ref. 20.

USING PDMP IN *IN VIVO* STUDIES

PDMP has been used in studies on mice and rats with interesting results. The administration of the inhibitor, however, requires different preparation. The acetate salt is more soluble than the hydrochloride in isotonic saline and can be dissolved at a reasonable concentration (1%) on heating to ~50°C. Because crystal formation does not begin immediately on cooling, one can inject the solution at body temperature. For convenience, we now use the hydrochloride the same way, frequently with inclusion of a nonionic detergent, Myrj 52, which helps solubilize the inhibitor (9). Myrj 52 is poly(oxyethylene) with ~40 oxyethylene residues, esterified in part with one or two stearate residues. It does not form peroxides, unlike most polyethylene glycol-based detergents, because it is solid. Later work with Myrj 52 showed some interference with DNA synthesis in cultured cells at a low cell density (10), as well as deposits in mouse liver. It is available on a sample basis from ICI Americas Inc. (Wilmington, DE 19897). Other detergents were not tried and it is possible that other dispersing agents might be suitable.

The concentrated solution of PDMP · HCl (for animal injection) is surprisingly acidic and we now buffer it to some extent by adding 8 mg of NaOAc/ml of solution (6 mg PDMP · HCl/ml + 12 mg Myrj/ml). DL-PDMP · HCl dissolves readily in the acetate–Myrj solution at 6 mg/ml.

Preliminary experiments with PDMP · HCl, at 30 mg/g in pulverized mouse chow, seemed to show that it readily enters the body by the oral route. Piperonyl butoxide was also added to the chow to block destruction of PDMP by cytochrome P-450 (see below). The chow was mixed with ethanolic PDMP and butoxide, dried under vacuum, mixed with additional powdered chow, and added to the feeder jars. The mice seemed to find the mixture quite attractive and no abnormal response was seen. This mode of administration (possibly following an initial injected dose) may yield more prolonged levels of the drug and might be particularly useful in experiments with tumors of the gastrointestinal tract.

If PDMP is injected after prior injection of piperonyl butoxide (dissolved in vegetable oil), its dosage level can

be lowered so that it may be unnecessary to include Myrj with the PDMP. This point needs further study.

Depending on the dose of PDMP, mice and some rats at times respond to intraperitoneal injections by showing hind leg weakness. Some exhibit excitation, rearing at the cage walls and switching their tails back and forth. Jaw motions, resembling a biting action, have been seen. These effects typically vanish in ~15 min and seem to have no permanent residua. Some of these symptoms were produced also by the ketonic form of PDMP (compound **III**) and sedation could be achieved with chlorpromazine (28). Curiously, chlorpromazine alone also lowered the level of liver GlcCer in mice and its use in future experiments with PDMP seems warranted.

Initial trials with mice injected with [^{3}H]PDMP at a typical dose (80 mg/kg) showed that much of the radioactive material in liver and kidney consisted of polar products that partitioned into the polar, upper phase of a chloroform–methanol–water system (11). Presumably these were the products of hydroxylation and conjugation reactions. Brain and spleen did not contain these metabolites but urine contained them almost exclusively. It seemed likely that the products originated in liver and kidney and were rapidly excreted. It was thus necessary to isolate the unmetabolized PDMP by TLC to determine the true concentrations of the inhibitor.

The injected inhibitor was found to enter the blood and organs very rapidly, with brain showing a slight lag. The maximal concentration of PDMP in liver and kidney was ~170 nmol/g of tissue, which corresponds to ~218 μM, a concentration that should have blocked GlcCer synthesis completely. Spleen demonstrated a lower level, ~140 nmol/g, and brain showed the lowest maximum, ~65 nmol/g. Even in brain, the glucosyltransferase must have been completely inhibited.

The PDMP concentrations in various mouse tissues dropped to near zero in 3 h. Thus, whatever the effects PDMP produced, they were produced in a short time. Accordingly, we investigated the possibility of blocking the degradative reactions, which are presumably due to one or more forms of cytochrome P-450. Piperonyl butoxide, a derivative of methylenedioxybenzene, has been used commercially in insect sprays for many years to block the P-450(s) in insects, in order to slow their inactivation of insecticides. Reports of much animal testing indicated that it is relatively nontoxic, so we injected the mice with 600 mg/kg dissolved in oil. Four hours later, we injected them with 80 mg/kg of PDMP · HCl in Myrj–saline. This yielded much higher levels of PDMP in the organs studied, with a maximum of 540 nmol/g of kidney. High levels were maintained for at least 16 h, but all of the drug was gone by 24 h. Less protection was obtained with another P-450 inhibitor, cimetidine.

More work is needed to establish the optimal doses of the piperonyl butoxide and PDMP, particularly because of the known tendency of substrates and inhibitors to induce P-450. It is likely that this induction effect could be blocked by semicontinuous application of the butoxide, perhaps in food. Although some isozymic forms of P-450 are required for certain normal metabolic conversions, as well as for the conversion of certain pro-drugs to their active forms, blockage by piperonyl butoxide is notable because of its small biological effects (thus far observed). It would seem advisable to test PDMP in future animal studies in conjunction with this inhibitor or alternatives such as fluconazole or ketoconazole. It might also be helpful to block the degradation of neuroactive drugs.

Piperonyl butoxide (technical grade, 90%) is sold by Aldrich Chemical and is listed in their catalog as "highly toxic," but numerous studies have reported the opposite properties. The LD_{50} for rats is very high, possibly 7 g/

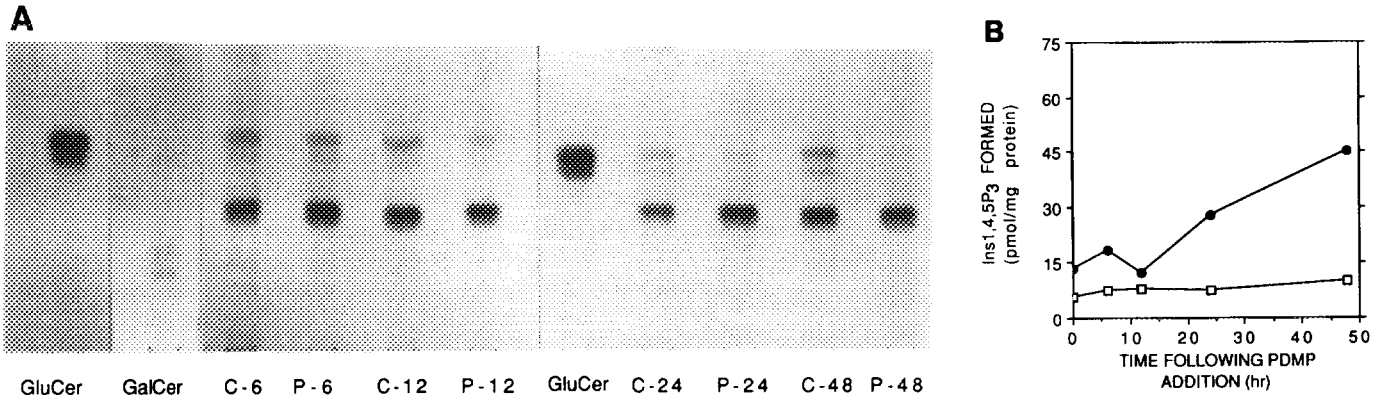

FIG. 4. Time-dependent association between PDMP exposure, accumulation of glucosylceramide (GlcCer), and $InsP_3$ formation. MDCK cells were exposed to control medium or to medium containing 20 μM PDMP for 6, 12, 24, or 48 h. (A) Charred TLC plates showing cerebroside amounts. (B) $InsP_3$ formation by isolated plasma membranes following exposure to GTPγS and bradykinin (●) or buffer alone (□) as described in the text. C denotes control conditions; P denotes PDMP-exposed cells. The data represent the mean of duplicate determinations. Reproduced from the *Journal of Clinical Investigation*, 1993, Vol. 91, pp. 797–803; by copyright permission of the American Society for Clinical Investigation.

kg body wt. We have seen no detectable toxicity at 600 mg/kg when the butoxide is administered ip diluted in corn oil or canola oil (15:85, v/v). The administration of other drugs (besides PDMP) that are attacked by cytochrome P-450 could cause adverse reactions if the butoxide slows their degradation. Thus, there is a need to redetermine the dose response toward coadministered drugs in P-450-depleted animals. It is likely that storage of the butoxide (a liquid ether) under air produces toxic peroxides and we recommend keeping it at −20°C.

An interesting observation in the drug metabolism study is that a small portion of the [^{3}H]PDMP, ~1%, was oxidized at the C-1 position, forming labeled water. In addition, the level of GlcCer synthase, assayed in tissue homogenates, decreased for a while after the single piperonyl butoxide–PDMP dose (at the 3- and 8-h points); by 24 h the specific activity of the enzyme was above normal. (It may be noted that this decrease in activity was not due to the presence of injected PDMP since the tissue was diluted 50-fold for assay and further dilution did not raise the activity.) These two observations suggest that some of the PDMP was oxidized to the ketone form (compound **III**), which covalently inactivated some of the synthase. Thus, PDMP appears to act by two mechanisms: by direct inhibition and by conversion to a second inhibitor. A third mechanism, observed with DL-PDMP in mice, involves elevation of GlcCer β-glucosidase activity via some unknown process (11). This change acts to lower the level of GlcCer more rapidly than one would expect from normal hydrolytic breakdown.

The problem of PDMP degradation may also be important in experiments with cultured cells that contain the P-450 microsomal oxygenase systems, such as hepatocytes. Inclusion of piperonyl butoxide should block P-450-dependent metabolism of PDMP. Only a few percent of both PDMP and PMMP were converted to new products by MDCK cells (10).

These considerations were recently applied to a study relating the GSL content of rat kidneys in animals made diabetic with streptozotocin (29). In this model of pharmacologically induced diabetes, an early response to hyperglycemia is the development of significant renal hypertrophy. Renal weights increase by 30% at 2 weeks and 90% by 2 months. The biochemical basis of this growth response is poorly understood. An observation in this model of diabetic nephropathy is the stimulation of the pentose phosphate pathway and the resultant accumulation of NADPH and UDP-glucose. Because NADPH is a cofactor for the synthesis of dihydrosphingosine (a precursor of ceramide) and UDP-glucose is a substrate for GlcCer formation, we considered the possibility that the level of tissue glucose under hyperglycemic conditions results in higher levels of renal GSLs. This was in fact observed to be the case. Significant increases in both GlcCer and ganglioside GM3 were observed in the diabetic kidneys compared to controls (Table 1). However, in animals made diabetic but then treated with insulin to normalize the blood glucose concentrations, no significant abnormality in GSL content was seen.

If, in the normal kidney, the concentration of UDP-glucose was well above the K_m for this substrate in GlcCer synthesis, the increase observed in the diabetic animals would appear to be of no significance. However, determination of the K_m in both normal and diabetic rats (250 μM in both) showed that the enzyme is normally not saturated with respect to this substrate. Thus, any increase in UDP-glucose concentration (Table 1) would yield the observed increase in GSLs of the diabetic rats. This observation gives further support to the belief that elevated levels of GlcCer stimulate organ growth and explains why depletion of GlcCer by PDMP causes impaired growth.

To demonstrate that the increase in renal GSL levels was functionally associated with the hypertrophic response, groups of normoglycemic or diabetic rats were followed for 2 weeks after the induction of diabetes. The rats were then treated with intraperitoneally administered PDMP (100 mg/kg body wt) and piperonyl butoxide (600 mg/kg) or with piperonyl butoxide alone for 3 days. Prior to sacrifice, the plasma glucose, blood urea nitrogen, and creatinine concentrations were assayed. The kidneys were weighed and studied by morphometric analysis. PDMP treatment had no significant effect on renal weight, glomerular volume, or plasma creatinine and BUN when compared to untreated controls. The diabetic animals treated with PDMP, however, exhibited normalization of both their renal weights and glomerular volumes when compared to the untreated diabetics (Table 2). There was no evidence of renal toxicity as assessed by renal histology and the renal function tests. These observations are consistent with the interpretation that GSLs mediate in part the pathophysiological consequences of diabetes in the kidney. It would be informative to assess whether similar

TABLE 1

Glycosphingolipid and UDP-Glc Levels in Diabetic and Insulin-Treated Diabetic Rats

	Control	Diabetic	Insulin-treated
GlcCer	29.4 ± 0.91	37.8 ± 1.42*	33.5 ± 1.21
Ganglioside GM3	43.9 ± 3.13	65.4 ± 6.46*	44.3 ± 12.5
UDP-Glc	78.4 ± 4.1	125 ± 7.3*	105 ± 8.14

Note. UDP-Glc levels are expressed as nmol/g wet wt of kidney. The data for GlcCer and ganglioside GM3 are expressed as μg/g wet wt. The GlcCer data represent densitometric measurements from the fastest moving cerebroside spot (representing cerebrosides not containing phytosphingosine or 2-hydroxy fatty acids), although all spots migrating as cerebrosides increased visibly in the diabetic kidneys. *$P < 0.05$ by analysis of variance and the Scheffé F test. The data are expressed as the mean ± SE ($n = 6$). Reproduced from the *Journal of Clinical Investigation*, 1993, Vol. 91, pp. 797–803, by copyright permission of the American Society for Clinical Investigation.

pathways are operative in other tissues susceptible to diabetic injury.

OTHER EFFECTS OF PDMP

Exposure of MDCK and other cultured cells to PDMP results in the accumulation of ceramide. Sphingosine and *N,N*-dimethylsphingosine were found to accumulate (18, 26). Presumably, free sphingol levels increase from the accumulated ceramide, by action of ceramidase. It is possible that transmethylation is also stimulated.

It is interesting to consider the two kinds of ceramide: acyl amides of sphingosine and acyl amides of sphinganine. The latter are, in part, converted to the former. Both are utilized for the formation of anabolites (mainly cerebrosides and sphingomyelin). The anabolites of ceramide consist largely (~90%) of derivatives of unsaturated ceramide, so it is likely that the latter are preferentially utilized by synthases. Any blockage of anabolite synthesis would be expected to result in the accumulation of the latter. In other words, the ceramides in a PDMP-treated cell are probably enriched in the unsaturated isomer. In a recent paper demonstrating that ceramide could produce programmed cell death (apoptosis), Obeid *et al.* pointed out that *N*-acetylsphingosine behaved like natural ceramides but *N*-acetylsphinganine was inactive (35). Thus, it is possible that PDMP, in elevating the level of unsaturated ceramide, is peculiarly effective in causing cell damage.

Diacylglycerol (DG) was also found to accumulate (26). This could be the result of activation of inositide hydrolysis, which yields DG as a co-product, but much of the isolated DG was found to resemble PtdCho in its fatty acid composition (27). Other experiments in this study supported the idea that this DG is formed by the action of PtdCho:ceramide phosphorylcholine transferase, the enzyme that catalyzes sphingomyelin synthesis.

PDMP-treated cells lost part of their ability to bind to matrix proteins (15, 16). This effect may play a significant role in the chemotherapy of cancer.

At relatively high concentrations in culture medium (>25 μM), DL-PDMP was found to partially inhibit sphingomyelin synthesis (12). The transport of a viral protein through the Golgi membranes, out to the cell periphery, was also slowed. Thus, it is possible that the high concentrations of PDMP attained (temporarily) in the animal studies affected glycoprotein and sphingomyelin synthesis. However, in cultured cells at the usual lower concentrations of PDMP, glycoprotein synthesis is unaffected. In the studies of mouse kidney lipids (9), sphingomyelin levels were unaffected by PDMP.

In view of all the above changes in important metabolites, it is not surprising that PDMP inhibits cell growth (protein synthesis) and DNA synthesis (thymidine incorporation). Yet the growth inhibition by PDMP, if produced slowly by low concentrations, does not produce cell death even when most of the GSLs have disappeared (19). This unexpected aspect has been discussed (30). It is possible that simple growth in the protected environment of culture dishes does not require the full complement of GSL molecules.

These biological effects of PDMP are more extensively discussed in recent reviews (31, 32).

APPLICATIONS TO NEUROCHEMISTRY

Since the nervous system contains significant amounts of GSLs (even though the major glycolipids are galactosphingolipids), it is now possible to look for behavioral changes in animals that have been depleted of GSLs with PDMP. There is, in addition, the possibility that PDMP would have therapeutic value in the treatment of brain damage due to trauma, strokes, etc. Gangliosides appear to be useful for treating various kinds of brain damage and it is interesting to note that they also inhibit GlcCer synthase fairly effectively (33). Ganglioside GD1a was the best inhibitor tested, inhibiting the enzyme by 55% at 40 μM. The suggestion was made that gangliosides act therapeutically—in part—by inhibiting GlcCer synthase, thus

TABLE 2

Characteristics of Control and Diabetic Rats Treated with PDMP

Group	*n*	Body wt (g)	Kidney wt (g)	Blood glucose (mM)	BUN (mg/dl)	Creatinine (mg/dl)
Control	12	241 ± 16	2.30 ± 0.17	10.1 ± 0.83	15 ± 2.4	0.28 ± 0.07
Control + PDMP	11	238 ± 21	2.25 ± 0.27	9.4 ± 0.89	13 ± 3.2	0.31 ± 0.09
Diabetic	7	203 ± 23	2.62 ± 0.29*	25.9 ± 3.22	24 ± 5.7	0.23 ± 0.05
Diabetic + PDMP	7	200 ± 11	2.32 ± 0.19	25.9 ± 4.33	28 ± 6.4	0.23 ± 0.05

Note. The data are expressed as the mean ± SD. All animals received piperonyl butoxide (600 mg/kg) intraperitoneally. Treated control and diabetic animals received DL-PDMP. *$P < 0.001$ by one-way analysis of variance using the Scheffé test. Reproduced from the *Journal of Clinical Investigation*, 1993, Vol. 91, pp. 797–803, by copyright permission of the American Society for Clinical Investigation.

slowing the growth and repair of glial cells. The latter may overgrow damaged neurons and block *their* repair, so it could be useful to inhibit glial growth by either gangliosides or PDMP. The latter is much more economical. It is likely that glial growth is dependent on GSL synthesis by adjacent neurons. Glia appear to lack GlcCer synthase (34) as well as some other GSL synthases and must acquire their GSLs by transfer from neurons.

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