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SHORT COMMUNICATION

Studies of the action of ceramide-like substances (D- and L-PDMP) on sphingolipid glycosyltransferases and purified lactosylceramide synthase

SUBROTO CHATTERJEE[1]*, TAVIA CLEVELAND[1], WAN-YANG SHI[1], JIN-ICHI INOKUCHI[2] and NORMAN S. RADIN[3]

[1]*Johns Hopkins University, School of Medicine, 600 N. Wolfe St, CMSC 604, Baltimore, MD 21287-3654, USA*
[2]*Seikagaku Corp., Tokyo Institute, Higashiyamato, Tokyo 207, Japan*
[3]*Nephrology Division, University of Michigan Medical School, Ann Arbor, MI 48109-0676, USA*

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We have studied the effects of D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) and its L-enantiomer on glycosphingolipids in cultured normal human kidney proximal tubular cells. We found that D-PDMP exerted a concentration-dependent reduction in the metabolic labelling and cellular levels of glucosylceramide (GlcCer), lactosylceramide (LacCer), and the globo-series glycosphingolipids, $GbOse_3Cer$ and $GbOse_4Cer$. It also directly inhibited the activity of UDP-glucose:ceramide $\beta 1 \rightarrow 4$-glucosyltransferase (GlcT-1) and UDP-galactose: GlcCer $\beta 1 \rightarrow 4$ galactosyltransferase (GalT-2). In contrast, L-PDMP had opposite effects on the metabolic labelling of GlcCer, LacCer, and $GbOse_3Cer$. The levels of GlcCer and LacCer were increased, while the labelling and level of $GbOse_4Cer$ were strongly reduced. Purified GalT-2 from human kidney was inhibited by D-PDMP and stimulated by L-PDMP. It appears likely that the different glycosphingolipid glycosyltransferases possess similar binding sites for the ceramide moiety, which are blocked by binding to D-PDMP and, in the case of $GbOse_4Cer$ synthase, by L-PDMP as well. The stimulatory effects of L-PDMP on GlcCer and LacCer synthases may be the result of binding to a modulatory site on the glycosyltransferases; in intact cells, the enzyme-analog complex may afford protection against the normal catabolic inactivation of the enzymes.

Keywords: PDMP (1-phenyl-2-decanoylamino-3-morpholino-1-propanol), glucosylceramide, lactosylceramide, globotriaosylceramide, globotetraosylceramide, inhibition and stimulation by PDMP enantiomers

Abbreviations: GalT-2, UDP-galactose:GlcCer β-galactosyltransferase; $GbOse_3Cer$, $Gal\alpha 1 \rightarrow 4Gal\beta 1 \rightarrow GlcCer$; $GbOse_4Cer$, $GalNAc1 \rightarrow 3Gal\alpha 1 \rightarrow 4Gal\beta 1 \rightarrow GlcCer$; GlcCer, glucosylceramide; GlcT-1, UDP-glucose:ceramide β-glucosyltransferase; GSLs, glycosphingolipids; LacCer, lactosylceramide; PDMP, threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol.

Introduction

Glycosphingolipids (GSLs) are complex sugar-containing sphingolipids. Recently, these compounds have drawn increased attention because of their possible functional roles in cell proliferation, cell differentiation and programmed cell death [1–3]. GSLs are composed of a sphingol (a long-chain sphingoid base), a fatty acid, and a simple or complex carbohydrate moiety. The hydrophobic moiety of GSLs, ceramide, is composed of the sphingol substituted at its amino group by a fatty acid in amide linkage. The sequential transfer of carbohydrate moieties from nucleotide sugars to ceramide gives rise to GSLs [4]. For example, the transfer of glucose from UDP-glucose to ceramide gives rise to glucosylceramide (GlcCer), which

*To whom correspondence should be addressed.

serves as the base for several hundred GSLs. The enzyme that catalyzes this transfer is termed GlcT-1. The enzyme that transfers galactose from UDP-galactose to GlcCer to produce lactosylceramide (LacCer) is termed GalT-2. Our laboratory has reported the purification of GalT-2 from human kidney [5].

Both GlcCer and LacCer have been shown to induce proliferation in a variety of cells [1, 6]. For example, the effects of GlcCer on the proliferation of a continuous cell line, MDCK, have been shown [6]. GlcCer enhanced epidermal proliferation [7] and, in intact mice, liver proliferation [8]. Previously we showed that, besides these two simple GSLs, other GSLs either moderately stimulated the proliferation of aortic smooth muscle cells or did not stimulate proliferation at all [1]. Most of the gangliosides investigated in our previous study did not stimulate the proliferation of cells, rather they caused a decrease in proliferation, possibly because they inhibit the biosynthesis of GlcCer [9].

Other studies have shown the reverse effect: reduction of cell growth and levels of GlcCer and LacCer by using a synthetic inhibitor of GSL biosynthesis [1, 6]. Properties of the inhibitor, D-PDMP, have been reviewed recently [10, 11]. From kinetic data, it appears that the compound binds to a region near the UDP-glucose-binding site and to a second site that acts like an activity modulator.

L-PDMP, on the other hand, *stimulated* the proliferation of cultured aortic smooth muscle cells [1]. It was also found to increase the level of LacCer in B16 melanoma cells [12, 13]. Taken together, these findings suggest that LacCer, GlcCer or a derivative of these compounds is involved in the proliferation of normal cells and of cells participating in proliferative diseases, such as atherosclerosis, cancer, and polycystic kidney disease [1, 14]. In this paper, we have used both enantiomeric forms of PDMP to investigate the biochemical mechanisms of action of these ceramide analogs on the activities of GlcT-1 and GalT-2 in cultured human proximal tubular cells.

Materials and methods

Isotopes and chemicals

D-[6-^{3}H]galactose (sp. act. 60 Ci mmol^{-1}, UDP-[^{14}C]glucose (sp. act. 9.25 Gbq mmol^{-1}), and UDP-[^{14}C]galactose (sp. act. 12.2 Gbq mmol^{-1}) were from Amersham Corp. and silicic acid (UnisilR) was from Clarkson Co., Williamsport, PA. Initial samples of D- and L-PDMP were furnished by the two last authors of this paper; later work was done with the materials from Matreya, Inc., Pleasant Gap, PA. Organic solvents were from J. T. Baker except for tetrahydrofuran, which was from Eastman, and redistilled just prior to use. Liquiscint was from National Diagnostics, Somerville, NJ. All other chemicals used were from Sigma, St. Louis, MO.

Cell culture

Normal proximal tubule cells were prepared from human kidney. Cells (10^5) were seeded in plastic Petri dishes (8 cm diam.) in 10 ml of medium containing 10% fetal bovine serum. The medium was changed on the third day. On the sixth day, the medium was removed and fresh medium containing lipoprotein-deficient human serum (1 mg of protein per ml) plus D-PDMP or L-PDMP was added as described in the figures and legends. The lipoprotein-deficient serum was used because whole serum stimulates GSL synthesis.

One set of dishes was incubated with [^{3}H]galactose (5 μCi ml^{-1}) for 24 h and harvested. These radiolabelled cells were used to investigate the incorporation of galactose into GSLs as described previously [15]. A second set of cells, incubated with or without D- or L-PDMP for 24 h, was harvested in phosphate-buffered saline and centrifuged. The cell pellets were utilized for the measurements of GSLs levels following extraction, purification, perbenzoylation and high performance liquid chromatography [16]. The third set of cells was harvested in cacodylate buffer (pH 6.8) as described [4], and the cell extracts were then utilized for the measurement of activity of GlcT-1 and GalT-2 after a 2-h incubation as described [17].

Three dishes were pooled for each analysis and at least nine dishes were used for each variable tested. The error bars in each figure represent the standard deviation of each group.

Results

Effects of D- and L-PDMP on the incorporation of [^{3}H]galactose into GSLs

We found that D-PDMP exerted a concentration-dependent inhibition of the incorporation of [^{3}H]galactose into GlcCer (Fig. 1A). The effect became noticeable at 5 μM inhibitor. D-PDMP also markedly reduced the incorporation of [^{3}H]galactose into LacCer, $GbOse_3Cer$, and $GbOse_4Cer$ (Fig. 1B–D). The latter decreases can be attributed, in part at least, to depleted levels of their precursor, GlcCer. However the decreases were seen with 2.5 μM PDMP, which did not affect GlcCer radioactivity, suggesting the possibility of direct influences of D-PDMP on the synthases that form the higher GSLs, such as GalT-2.

In contrast, L-PDMP stimulated the incorporation of [^{3}H]galactose into GlcCer, LacCer and globotriaosylceramide (Fig. 1A–C). L-PDMP at 5–20 μM stimulated the incorporation of [^{3}H]galactose into GlcCer ~50% compared to controls. The stimulations in the incorpora-

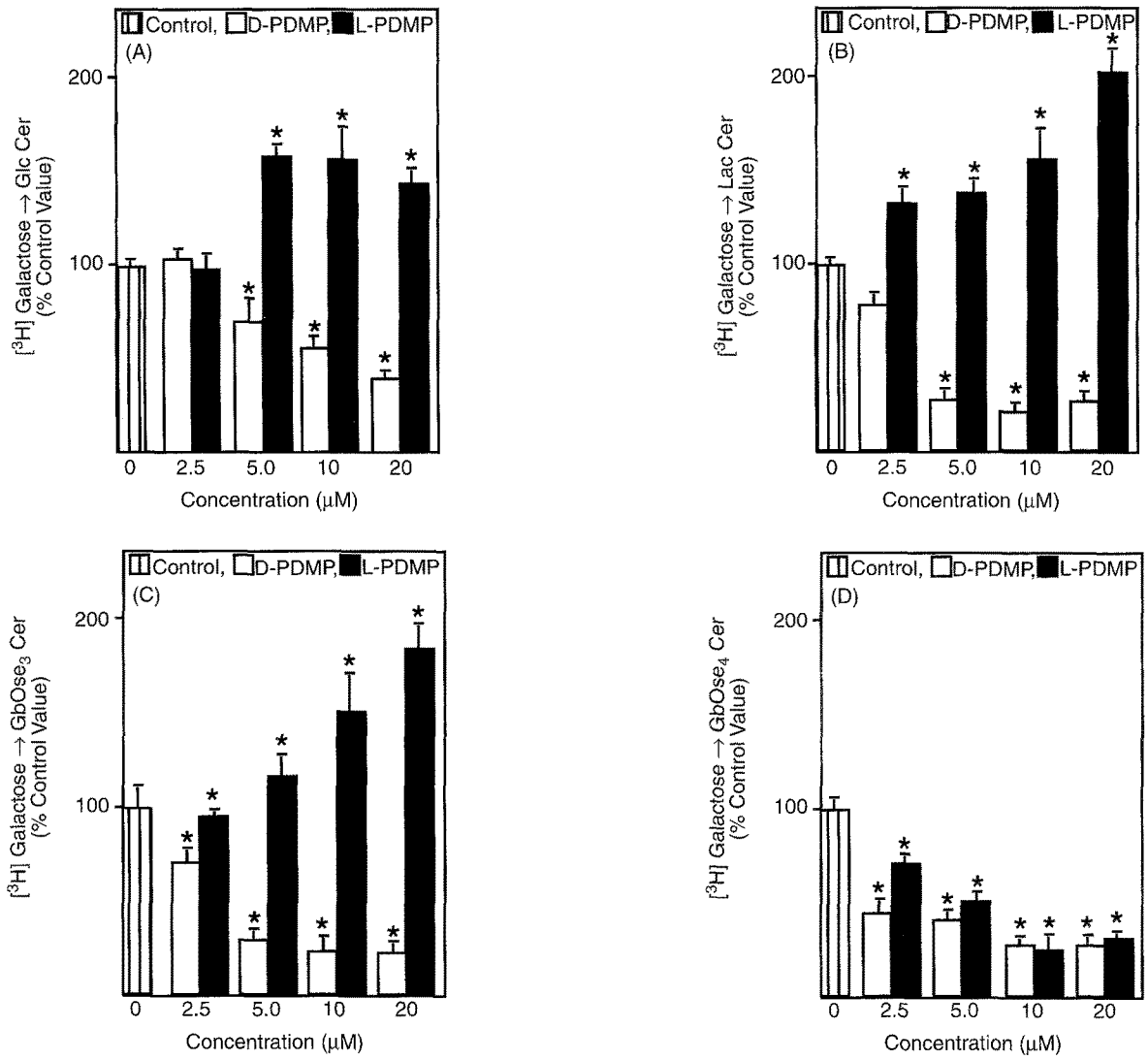

Figure 1. Effects of D-PDMP and L-PDMP on the incorporation of [^{3}H]galactose into various glycosphingolipids in cultured normal human proximal tubular cells. Cells (10^5) were seeded in 8 cm plastic Petri dishes. After they reached confluence, the medium was replaced with fresh medium containing lipoprotein-deficient serum, [^{3}H]galactose (5 μCi ml^{-1}), and D-PDMP or L-PDMP (0–20 μM). Incubation was continued for 24 h. Next, the cells were harvested, GSLs were isolated, and the incorporation of [^{3}H] into individual GSLs was measured after separation on HPTLC plates and scintillation spectrometry. The data are reported as cpm per mg protein. The control specific activities were: (A) GlcCer, 150 ± 15; (B) LacCer, 810 ± 75; (C) $GbOse_3Cer$, 11,550 ± 1,444; and (D) $GbOse_4Cer$, 4, 102 ± 450. The protein contents of the dishes in the three sets were 580 ± 51 μg in the control dishes, 510 ± 25 μg in the D-PDMP dishes, and 590 ± 60 in the L-PDMP dishes. Asterisks above graph columns here and in the following figures show values that are statistically different ($p < 0.005$) from the control values.

tion of [^{3}H] into LacCer and $GbOse_3Cer$ were even greater, and dose-dependent (Fig. 1B, C). Thus the possibility is raised that L-PDMP stimulated the synthases forming LacCer and $GbOse_3Cer$, in addition to GlcCer synthase.

In a striking demonstration of the specificity of L-PDMP action, the compound produced a marked *decrease* in the incorporation of [^{3}H]galactose into $GbOse_4Cer$, even at 2.5 μM (Fig. 1D). We may speculate that the L-isomer acts to directly inhibit the *N*-acetylgalactosaminyltransferase that forms $GbOse_4Cer$. The same can be said for D-PDMP, which also strongly inhibited the incorporation of [^{3}H]-Gal into $GbOse_4Cer$, more so than into its precursor.

Effects of D-PDMP and L-PDMP on the cellular levels of GSLs

D-PDMP produced marked decreases in the concentrations of GlcCer, LacCer, $GbOse_3Cer$, and $GbOse_4Cer$. Such decreases in GlcCer and LacCer have previously been seen in a variety of cell types and they are evidently due, in part at least, to depletion of GlcCer stores by the direct

inhibitory action of D-PDMP on GlcCer synthase. Presumably all the GSLs are in a dynamic state, undergoing hydrolysis as well as synthesis; thus they decrease in concentration as the supply of GlcCer decreases. The decreases in $GbOse_3Cer$ and $GbOse_4Cer$ have not been reported before. The most remarkable effect of D-PDMP was on the cellular levels of $GbOse_3Cer$, acting at concentrations <2.5 μM. This difference suggests that D-PDMP directly inhibits $GbOse_3Cer$ synthase.

As might be expected from L-PDMP's effects on [^{3}H]galactose incorporation into the GSLs (Fig. 1), the ceramide analogue markedly stimulated the cellular levels of GlcCer, LacCer and (to a small extent) $GbOse_3Cer$, but reduced the cellular levels of $GbOse_4Cer$ (Fig. 2). The cellular level of GlcCer was increased approximately 100% with 10 μM L-PDMP. At higher concentrations, the levels of GlcCer were decreased but still about 60% higher than the control value. At higher concentrations (20 μM), the stimulatory actions of L-PDMP were lessened, suggesting the possibility that the compound acts at two sites, one stimulatory, the other inhibitory.

Effects of L- and D-PDMP on the activity of GlcT-1 and GalT-2 in cell extracts

D-PDMP exerted a concentration-dependent inhibition of the specific activity of GlcT-1 in the crude extracts from normal cells (Fig. 3A). Distinct inhibition was seen at 2.5 μM inhibitor and the activity was decreased to about

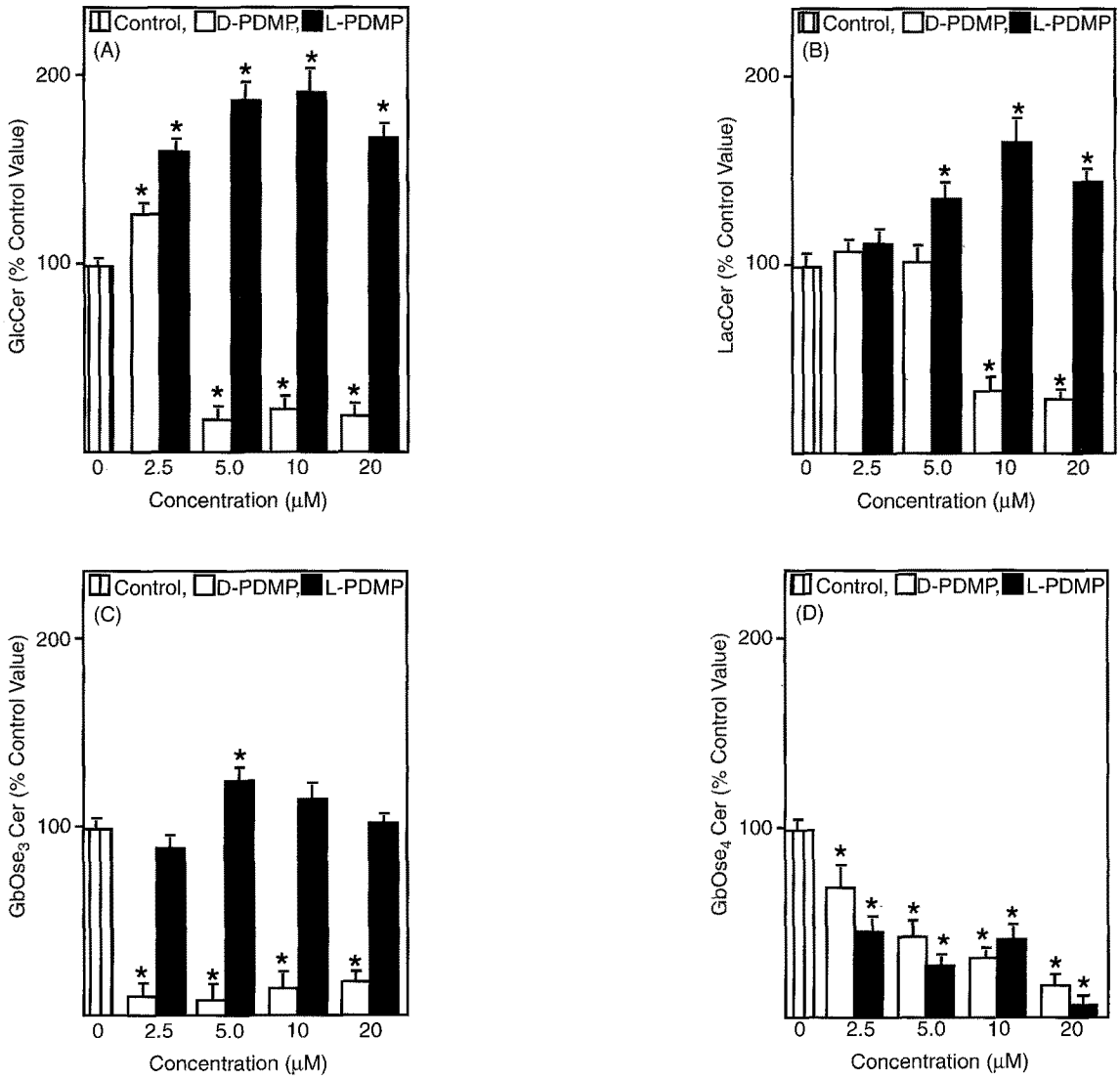

Figure 2. Effects of D-PDMP and L-PDMP on the cellular levels of glycosphingolipids in cultured proximal tubular cells. The protocol described in Fig. 1 was followed except that the cells were incubated without [^{3}H]galactose. Following incubation and harvesting, the GSLs were isolated and quantified by high performance liquid chromatography after perbenzoylation. The control values (nmol/mg protein) were: (A) GlcCer, 0.7 ± 0.07; (B) LacCer, 0.3 ± 0.03; (C) $GbOse_3Cer$, 1.8 ± 0.15; (D) $GbOse_4Cer$, 1.1 ± 0.1. The protein contents of the three sets of dishes were 530 ± 65 μg (controls, 485 ± 45 μg (D-PDMP), and 554 ± 70 μg (L-PDMP).

30% of normal at 10 μM. This observation adds normal human kidney cells to the list of cells whose GlcT-1 is directly inhibited by PDMP. L-PDMP stimulated the activity of GlcT-1 in these cell extracts, in agreement with our observations of [^{3}H] incorporation and cell GlcCer levels.

We found that D-PDMP also decreased the activity of GalT-2 in these enzyme preparations (Fig. 3B), although the effect was weaker than that seen with GlcT-1. On the other hand, L-PDMP markedly stimulated the activity of GalT-2, the effect being stronger than that seen with GlcT-1. The maximal stimulation, at 10 μM, was 80% above the control activity.

Effects of D- and L-PDMP on purified GalT-2 from human kidney

Evidence that the above-described effects of PDMP on purified GalT-2 in broken cell preparations represented direct action on the enzyme (rather than indirect effects involving other cell components) was obtained by tests with purified GalT-2. We found that D-PDMP strongly inhibited GalT-2, even at a concentration of 1 μM (Fig. 3C). The use of higher D-PDMP concentrations did not lower the activity any further.

In contrast, L-PDMP markedly stimulated the activity of GalT-2 in the purified preparation. Here too the effect was visible at a low concentration. However, at higher concentrations (10–20 μM) L-PDMP acted as an inhibitor, again indicating opposing actions at two different enzyme sites (Fig. 3c).

Discussion

This study revealed several major findings. D-PDMP, initially thought to inhibit only GlcCer synthase, was found to directly inhibit LacCer synthase (GalT-2). Suggestive evidence was also found for a direct inhibitory action on the two globo-series synthases. These effects are in agreement with recent data from the Inokuchi laboratory [13], which found direct inhibition in B16 melanoma cells of GlcT-1, GalT-2, and the sialyltransferase that converts LacCer to ganglioside GM3. This rather

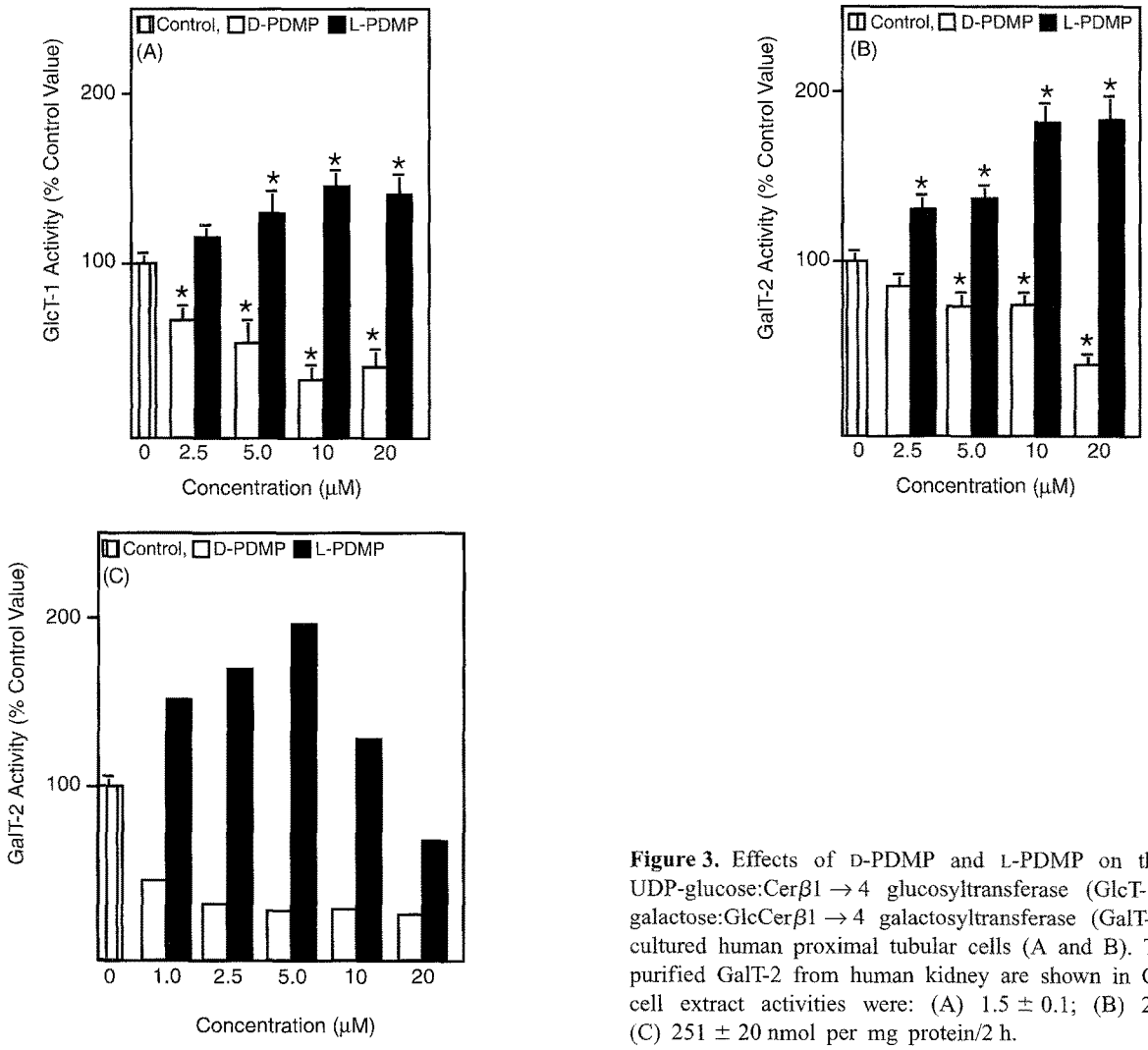

Figure 3. Effects of D-PDMP and L-PDMP on the activity of UDP-glucose:Cerβ1 $\rightarrow$ 4 glucosyltransferase (GlcT-1) and UDP-galactose:GlcCerβ1 $\rightarrow$ 4 galactosyltransferase (GalT-2) activity in cultured human proximal tubular cells (A and B). The effects on purified GalT-2 from human kidney are shown in C. The control cell extract activities were: (A) 1.5 $\pm$ 0.1; (B) 2.5 $\pm$ 0.2; and (C) 251 $\pm$ 20 nmol per mg protein/2 h.

general inhibitory action of D-PDMP is probably due to the presence of a similar binding site common to all these enzymes. At the present time we can only speculate that the ceramide-binding site, probably underwent little evolutionary mutation during the development of GSLs with more complex structures. The enzymes forming more complex GSLs, such as the ganglioside synthases, probably appeared later in evolutionary history, so their ceramide-binding regions may have mutated to regions that bind D-PDMP more weakly. The essential role of ceramide moiety in GalT-3 a galactosyltransferase that catalyzes the transfer of galactose form GM2 to GM1 has been reported [18]. This hypothesis raises the possibility that analogs of PDMP could be synthesized with more specific ability to inhibit the more complex synthases. Several new analogues have recently been described [19]; some of them are much more inhibitory against GlcT-1 and it will be interesting to compare the enantiomeric forms against the higher glycosyltransferases.

The stimulatory actions of L-PDMP reported here are consistent with the report that it increased the level of GlcT-1, GalT-2, and LacCer sialyltransferase in melanoma cells [13]. However those effects could be demonstrated only in growing cells, not in cell lysates, and it would appear that the phenomenon involved was inductive in nature. Culturing cells exposed to cycloheximide caused them to lose most of their GalT-2 activity, indicating that this enzyme normally undergoes rapid synthesis and inactivation. In the presence of L-PDMP, however, the loss of enzyme was not as great. This may signify that L-PDMP binds to the enzyme, stabilizing it against the normal catabolic inactivation process. In the case of our purified GalT-2, the stimulation by L-PDMP was direct, not inductive.

The especially strong reduction in synthesis of the globo-series GSLs reported in our study raises the possibility that a mixture of D- and L-PDMP would be particularly effective in blocking the binding of various pathogenic organisms [20, 21]. These organisms must bind to globoside-bearing sites on human and porcine cells in order to produce their toxic actions. DL-PDMP has been shown to successfully block such binding between *E. coli* and human kidney and intestine cells *in vitro* [22]. Certain human tumours have also been shown to be enriched in globosides and it is possible that they are particularly sensitive to DL-PDMP.

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