Metabolism of D-[^{3}H]*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, an inhibitor of glucosylceramide synthesis, and the synergistic action of an inhibitor of microsomal monooxygenase

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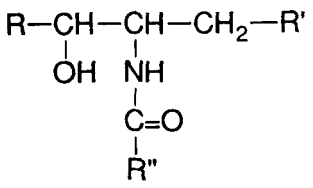
Abstract D-*Threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) is an effective inhibitor of the glucosyltransferase that makes glucosylceramide. Virtually all of the hundreds of naturally occurring glycolipids are formed from this primary glycolipid, so the inhibitor acts to lower their concentrations by the process of attrition (hydrolytic catabolism). Trials with mice carrying ascites carcinoma cells showed that PDMP could produce a permanent cure in some of the animals and marked prolongation of life in the others (Inokuchi, J., I. Mason, and N. S. Radin. 1987. *Cancer Lett.* **38:** 23–30). In order to maximize the effect, we studied the metabolism of PDMP by labeling it with [^{3}H] on carbon one, using a labeling method that discriminated against the unwanted *erythro*-isomer. The active enantiomer of the inhibitor (D-) was isolated by chromatography of the camphanate esters, followed by methanolytic cleavage. Examination of the fate of the labeled drug after a single injection showed that it was very rapidly converted to several polar products that were rapidly excreted. The drug penetrated all of the organs readily and a small portion was oxidized at the C-1 position to yield ^3H_2O. From these findings it appeared likely that the amine is attacked by a mixed function oxidase based on cytochrome P450. This conclusion was confirmed by showing that the tissue levels of PDMP could be greatly elevated, for a much longer time, when the mice were pretreated with piperonyl butoxide or cimetidine. The amount of conversion to polar metabolites was substantially reduced and tissue levels of PDMP were maintained much longer. Analysis of mice injected with one or both drugs showed that piperonyl butoxide augmented the effects of PDMP on ceramide, glucosylceramide, and dihexosylceramide levels, as well as on the activity of glucosylceramide synthase. It is suggested that piperonyl butoxide be used as an adjuvant for the many useful drugs that are inactivated by the P450 system. —**Shukla, A., and N. S. Radin**. Metabolism of D-[^{3}H]*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, an inhibitor of glucosylceramide synthesis, and the synergistic action of an inhibitor of microsomal monooxygenase. *J. Lipid Res.* 1991. **32:** 713–722.

Supplementary key words monooxygenase inhibition • liver • kidney • ceramide metabolism • ceramide dihexoside

Glucosylceramide (GlcCer), the precursor of a large number of glycosphingolipids, is synthesized from N-acyl sphingols (ceramides) and UDP-glucose by a glucosyltransferase, EC 2.4.1.80 (1). The enzyme can be inhibited efficiently by a cationic lipid, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), that resembles the structures of both ceramide and its product, GlcCer (2) (see **Fig. 1**). The morpholine ring can be considered to be a cationic analog of the glucopyranosyl ring. In another respect, it resembles the commonly used pharmaceutically active diethylamino group. Only one of the four enantiomeric forms of PDMP is active in the enzyme assay; it appears to be the D-*threo* isomer (3). The inhibitor has shown promise in the treatment of mice bearing the Ehrlich ascites carcinoma (4) as well as in mice and rats bearing other transplantable tumors (G. S. Shukla and A. Shukla, unpublished results). In vitro, it has produced glycolipid-deficient melanoma cells that show impaired adhesion to laminin and collagen IV (5), and Lewis lung carcinoma cells with depressed ability to metastasize (6). It has also produced T cells that could not proliferate after exposure to interleukin 2 (7) and kidney cells whose phosphatidylinositol diphosphate phosphatase was especially sensitive to bradykinin (8). Mice injected with PDMP have developed rapid decreases in tissue levels of GlcCer and increases in the activities of glycolipid hydrolases (9).

In view of the possibility of developing practical application of D-*threo*-PDMP to the treatment of cancer, gly-

Abbreviations: GlcCer, glucosylceramide (glucocerebroside); GSL, glucosphingolipid; PB, piperonyl butoxide (90% pure); PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; TLC, thin-layer chromatography; C, M, and W in solvent mixtures, chloroform, methanol, and water. Stahl's definition of hRf is 100 × R_f.



Glucosylceramide: R = CH_3-$(CH_2)_{12}$-CH=CH-

R' = β-glucopyranose

R" = $C_{15}H_{31}$ to $C_{24}H_{49}$

PDMP: R = phenyl

R' = morpholine

R" = C_9H_{19}

Fig. 1. Comparison of GlcCer and PDMP structures. Not shown: the hydroxyl group has the opposite (unnatural) configuration in D-*threo*-PDMP.

cosphingolipidoses, and other diseases in which glycolipids appear to play a significant role, it seemed important to investigate the metabolism and distribution/excretion behavior of the compound. To this end, we synthesized the inhibitor in ^{3}H-labeled form and studied its processing by mice. Since our findings indicated that the inhibitor is metabolized rapidly by cytochrome P450, we investigated the possibility of blocking this conversion by inactivating the microsomal monooxygenase system.

MATERIALS AND METHODS

Thin-layer chromatography

TLC was run with precoated silica gel 60 plates (E. Merck, #5763-7, 0.2 mm thick, 20 cm long), used without reactivation or washing. The spots were revealed by spraying 1% methanolic iodine or the fluorogenic spray, primulin (10 μg/ml in acetone–water 4:1, viewed at 365 nm) (10), or alkaline bromthymol blue spray. Solvent A was C–M–HOAc 90:10:10; solvent B was C–M–HOAc 90:5:10; solvent C was C–M–HOAc–acetonitrile 92:3:4:2.

Chemical synthesis

2-Decanoylamino-3-morpholino-propiophenone · HCl was synthesized from N-decanoylamino acetophenone (3) and recrystallized from 50 vols of hot ethyl acetate. (See synthesis scheme, **Fig. 2**.) The solution was allowed to cool slowly to room temperature over a 30-min period, with constant stirring. It was then stirred 1 h in a cold room and filtered through a sintered glass pressure funnel, rinsing with cold ethyl acetate. Drying in vacuum over P_2O_5 yielded the ketone (71% yield), migrating in TLC with solvent B at hRf 57. A fast-moving, faint impurity at hRf 92 was visible.

The carbonyl group was reduced with NaB[^{3}H]$_4$ (NEN Research Products, DuPont, 389 Ci/mol). A solution of 23.5 μmol of nonradioactive borohydride (0.89 mg in 17.8 μl of 5 mM NaOH) was added with a microsyringe to (nominally) 24 mCi of borotritide in its original rubber-stoppered shipping vial. To this solution was added a solution of 20 mg (47 μmol) of the ketone in 4 μl (47 μmol) of morpholine, 25 μl of water, and 106 μl of "reagent ethanol" (EtOH–MeOH–isopropanol 90:5:5, Fisher Scientific), also with a syringe. The reactants were mixed gently and left 1 h. This solvent was used because of its presumed lack of aldehydes.

Under these conditions the reduction was incomplete and TLC in trial runs showed that *threo*-PDMP was formed preferentially. Reduction was completed by adding 142 μl of the cold borohydride solution. Drops of oil formed at this stage, which were redissolved with 568 μl of "reagent ethanol." After 1 h, the reaction mixture was transferred to a large test tube with 7 ml (in portions) of MeOH–isotonic saline 4:3 and 8 ml of chloroform. The lower layer, containing DL-PDMP free base, was washed twice with 4-ml portions of MeOH–saline 1:1 and once with MeOH–W 1:1. The chloroform was added after the borohydride had been diluted to avoid chemical reaction.

The radioactive material in the lower layer was examined by TLC (solvent A) with carrier *threo*- and *erythro*-PDMP. The two PDMP spots, located with primulin, were scraped off the plate and counted after dissolving the silica gel in 0.3 ml of 49% HF and 0.4 ml of water (11). The lower (*threo*) spot contained 34% more activity than the faster spot (*erythro*). About 9% of the total activity on the plate appeared near the top, possibly due to reduction of contaminating decanoylaminoacetophenone. The total yield of [^{3}H]PDMP, based on the TLC spots, was about 1.16×10^9 cpm.

Resolution of DL-*threo*-PDMP and removal of DL-*erythro*-PDMP

The lower layer obtained by partitioning was evaporated to dryness in a 20 × 150 mm test tube and left over $CaCl_2$ in vacuo to ensure dryness. The residue, assumed to weigh 20 mg, was treated with 0.25 ml of a 10% solution of (1R)-(−)-camphanic acid chloride (2.5 mole equivalents) in anhydrous pyridine (kept in a sealed nitrogen-filled bottle. Aldrich #27,097-0). Pyridine stored under air, despite drying with molecular sieve, gave poor yields (Inokuchi, J-i., Fukuoka University, personal communication). After an overnight reaction period at 40°C, the solution was treated for 1 h with 0.2 ml of MeOH to destroy excess acyl chloride. The excess pyridine base was removed by rotoevaporation with toluene.

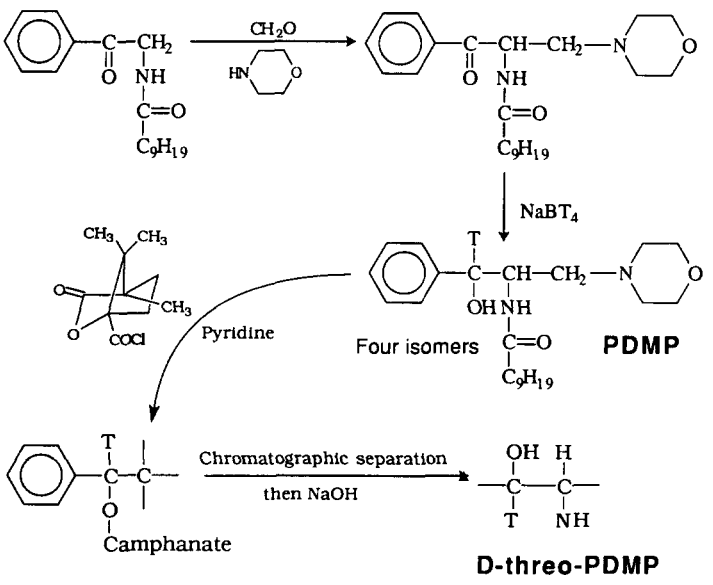


Fig. 2. Reaction sequence in synthesizing, isolating, and checking the purity of D-*threo*-[^{3}H]PDMP.

The pyridine·HCl formed from the acid chloride was removed by partitioning the residue in C-M-W 8:4:3 ml containing a trace of thymol blue. Conc. NH_4OH was added to produce a blue color and the lower layer was washed with 4 ml of M-W 1:1.

Analysis of the ester mixture by TLC with solvent C, together with carrier, yielded a minor fast spot at about hRf 36 and a major elongated spot just below it. Comparison with standards showed that the faster spot was due to the D-*threo* ester while the slower material was a fortuitous mixture of L-*threo* and DL-*erythro* esters. Thus it was possible to isolate the D-form without going through the procedure of removing the *erythro* isomer.

A preliminary purification was carried out to minimize contamination of the high-performance chromatography column used next for the isomer separation. The mixture of esters was promptly evaporated to dryness without using heat (to minimize hydrolysis) and redissolved in 0.5 ml of hexane–t-butyl methyl ether–acetonitrile–triethylamine 50:45:5:1. The solution was purified with a 0.5 × 33 cm column containing 2 g of silica gel 60 (Merck #9385, 230/400 mesh), packed in hexane, and equilibrated with the same solvent. The major labeled fraction, containing all four enantiomers, eluted from the column between 24 and 55 ml in a tritium yield from PDMP of ~87% (~10^9 cpm).

A mixed-mode (cation/C_8) 7-μm diameter reversed phase, 4.6 × 250 mm column was used next, together with a similar packing in a guard column (Alltech Associates). The column was operated with aqueous 20 mM K^+-PO_4 (pH 4)–acetonitrile–methanol 45:37:20 at 1 ml/min and 25-μl (100 μg) portions of purified esters were injected via a 0.2-ml loop. After every four or five runs, the column was washed with 100 ml of acetonitrile–water 1:1 to remove accumulating impurities. Monitoring at 264 nm revealed the elution of a small peak corresponding to the faster TLC spot, followed with slight overlapping by a larger peak. The two peaks were collected from multiple runs and pooled separately.

Methanolysis of the D-*threo*-PDMP camphanate

The effluent containing D-*threo*-PDMP camphanate was rotoevaporated to dryness without heat and the ester link was cleaved in 1 h with 12 ml of chloroform and 6 ml of 0.21 M methanolic NaOH. The NaOH was removed by partitioning with 4.5 ml of isotonic saline and the lower layer was washed several times with MeOH–saline 1:1 containing a trace of phenolphthalein until the upper layer was pale pink (at pH > ~ 10 the indicator is intense pink). No radioactivity was found in the upper layers and the yield of D-*threo*-PDMP tritium was 33%, as opposed to a hypothetical 25% of the four isomers. The specific activity was 10,760 cpm/nmol.

Purity confirmation

A test of the final product by TLC with a solvent A showed that 4% of the radioactivity was found in the faster (*erythro*-) PDMP spot, evidently due to slight overlap in the final chromatographic step. (Note that the camphanate ester of *threo*-PDMP migrates faster while the free base migrates more slowly than the corresponding *erythro* isomer). The enantiomeric purity was checked by mixing labeled and unlabeled DL-*threo*-PDMP, converting the sample to the camphanate as described above, and chromatographing the ester by TLC as above. A 40 μg spot containing 6240 cpm yielded a radioautogram that showed

(with the aid of EN^{3}HANCE) only a single labeled streak; that is, no L-*threo*-PDMP could be detected.

Animal experiments

The [^{3}H]PDMP, mixed with unlabeled PDMP·HCl to give 25.7 cpm/nmol, was dissolved at a concentration of 8 mg/ml in isotonic saline containing 12 mg/ml of the nonionic detergent, Myrj 52. Warming in hot tap water was necessary to dissolve the amine. Each experimental group consisted of five ICR male mice (Harlan Sprague-Dawley), selected with a computer program (12) in such a way that the groups had very similar mean body weights and distribution of weights around the mean (SD). All weights mentioned in this paper are wet weights.

Study 1. Each mouse, average weight 36.1 ± 1.2 g, was injected intraperitoneally with 80 mg/kg (~169,900 cpm) of D-*threo*-[^{3}H]PDMP. After injection, the mice were anesthetized with ether at intervals of 1/3, 1, 3, 8, and 24 h and blood was drawn from the heart, after which the organs were removed. The blood was drawn with an EDTA-wetted syringe and collected in tubes loaded with 0.2 ml of 1% EDTA. Blood from the orbital vein (25 μl) was drawn from the 1-h group 5 min after injection and from the 3-h group 10 min after injection. The 8-hr and 24-h groups were kept in metabolism cages to collect feces and urine. The liver, kidney, spleen, and brain were frozen over dry ice and stored at −70°C.

Study 2. Ten groups of five animals each (weight = 31.4 ± 1.1 g) were injected i.p. with piperonyl butoxide (600 mg/kg, 150 mg/ml corn oil) or with corn oil alone (4 mg/kg). No reactions to the injections were seen. Four hours later all mice were injected i.p. as before, with 80 mg/kg (130,000 cpm) of D-*threo*-PDMP in Myrj–saline.

Study 3. Groups of five mice each were injected with either peanut oil (50 μl i.m.) or PB (600 mg/kg in 50 μl peanut oil, i.m.) or cimetidine (200 mg/kg in saline, i.m.). PDMP was injected subcutaneously 4 h later into all mice at 40 mg/kg and the groups were killed 1, 3, or 16 h after PDMP injection.

Study 4. This was similar to Study 2 but the PDMP was not labeled and lipid analyses and enzyme assays were carried out. These mice weighed 28.0 ± 0.9 g. This time a Myrj–saline control group was included too, as well as a corn oil control group. Both groups of controls were killed 8 h after the initial 4-h injection, while the other groups were killed 3, 8, or 24 h after the last injection.

The tissues were analyzed for sphingolipid content by computer-controlled densitometry of TLC plates (9, 13) and glucosylceramide synthase was assayed by an improved method (14) that utilizes UDP-[^{3}H]glucose, an ultrasonic incubation bath, and a novel liquid/liquid partition system.

Counting tissues and extracts

In the case of brain, liver, and kidney, a 20% homogenate in water was prepared and 0.35 ml of this was left at 60°C for 1–2 h with 1 ml of Protosol (Tissue solubilizer TS-2 0.5 N, Research Products International). The samples were bleached for 30 min with 100 μl of 30% H_2O_2 at 60°C and diluted with 10 ml of CytoScint (ICN Biomedicals). After 30 min the samples were counted.

In the case of spleen, the whole spleen was minced and heated with Protosol (1 ml/200 mg) at 60°C overnight. Counting proceeded as above, with 0.2 ml of bleached digest.

Blood samples were treated with 0.5 ml of Protosol–ethanol 1:2, incubated at 60°C for 1 h, then bleached with 0.5 ml of 30% H_2O_2 as above. In this case, after adding the CytoScint, 0.5 ml of 0.5 N HCl was added (15).

The blood samples drawn at the time of killing were centrifuged at low speed and 50 μl of plasma was counted directly with CytoScint. The red cells were washed with saline and hemolyzed with an equal volume of water; 50 μl was treated like whole blood. The urine samples (0.5 ml) were counted directly with CytoScint.

Feces were converted to a 10% homogenate in water. A 1-ml portion was extracted with 33 vols of hexane–isopropyl alcohol (16) and 0.5 ml of the extract was evaporated to dryness, taken up in 0.5 ml of water and 10 ml of CytoScint, then counted.

Reagents

The reagents whose sources were not given were from Sigma Chemical or Aldrich Chemical. Standard sphingolipids were prepared here by previously published methods.

Statistics

Significance of differences was calculated by one-way ANOVA followed by the Scheffé F-test. Differences between control and experimental groups were considered significant if they matched or exceeded the 95% confidence level.

RESULTS

Partitioning, extractability, and stability tests

PDMP, as the free base, partitioned completely into the chloroform-rich layer of an alkaline C–M–W 8:4:3 mixture. When the pH was not controlled, as with a partition using PDMP·HCl and C–M–W, about 10% of the PDMP went into the upper layer.

Two 100-mg pieces of liver were each homogenized with 2.4 ml of hexane–isopropyl alcohol 3:2; one of the test

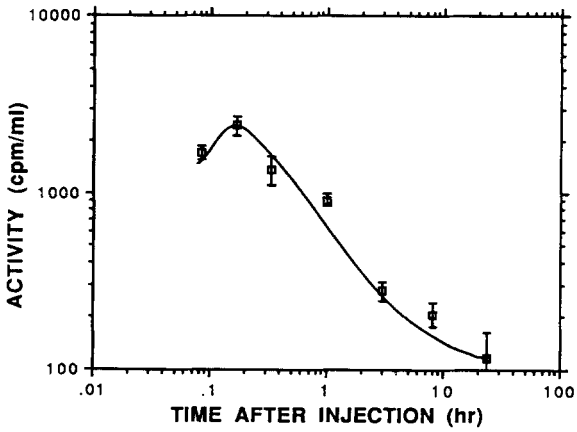


Fig. 3. Radioactivity in whole blood as a function of time after a single intraperitoneal injection of [^{3}H]PDMP into mice at 80 mg/kg. The error bars represent the SDs of the means.

tubes also contained 3000 cpm of [^{3}H]PDMP. After centrifugation, 97.6% of the radioactive material was found in the supernatant liquid, showing that there is little or no tissue material that binds the amine strongly.

Good stability of an aqueous solution of DL-*threo/erythro*-[^{3}H]PDMP·HCl (18,000 cpm), stored 24 h at room temeprature, was demonstrated by converting the salt to the free base, extracting the amines by the above partition method, and examining them by TLC with solvent A. Only the two spots for *threo*- and *erythro*-PDMP were seen with primulin, and counting of the silica gel showed good recovery in the spots (17,320 cpm or 96%) and no tritium in the other regions. This experiment also confirmed the efficacy of the HF-scintillation technique used (11).

Time study of tritium metabolism

The radioactivity in the blood rose rapidly, peaking at about 10 min after intraperitoneal injection (Study 1, **Fig. 3**). The loss from the blood was very rapid, with a half-life of ~50 min in the early period. No tritium was found in the red cells. The peak plasma tritium level corresponded to a concentration of 190 μM, assuming that the PDMP was the only labeled component.

The radioactivity in liver also rose very rapidly (**Fig. 4A**), the maximum observed value corresponding to 673 μM. (The molarity is calculated on a weight basis,

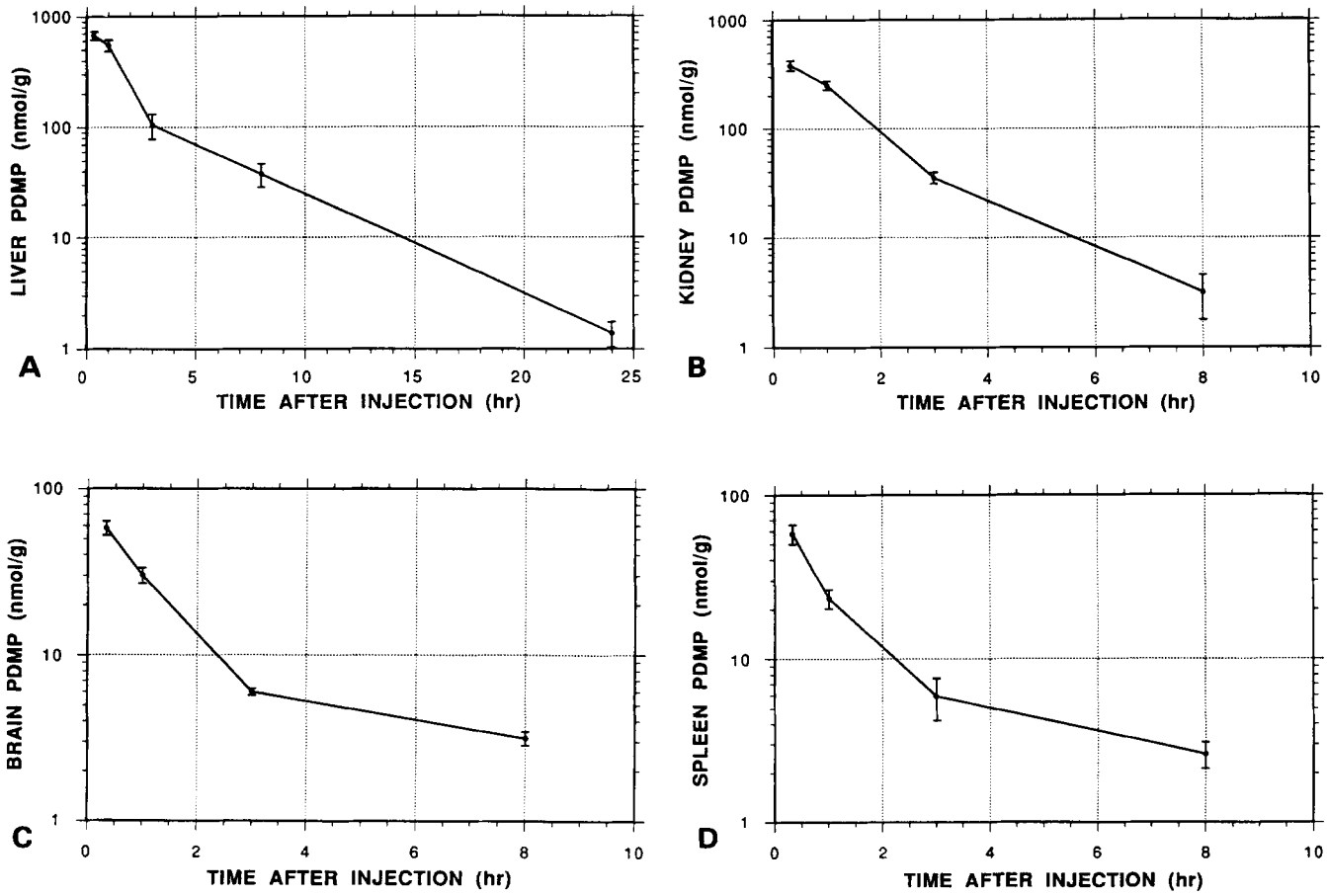


Fig. 4. Total radioactivity, in PDMP equivalents, as a function of time after a single intraperitoneal injection of [^{3}H]PDMP into mice at 80 mg/kg. The error bars show the SDs; 4A: liver; 4B: kidney; 4C: brain; 4D: spleen.

moles/kg of tissue.) This is much higher than the blood concentration. The tritium level decreased relatively slowly during the first hour but accelerated sooon after, with a half-life only a little longer than that seen in blood.

Kidney showed an excretion pattern like that of liver, with a maximal concentration of 380 μM and a $t_{1/2}$ of ~50 min (Fig. 4B).

Brain reached a maximal PDMP-equivalent concention at 20 min of 58 μM. It too showed two (or more) excretion phases (Fig. 4C). The pattern and activities in spleen were quite similar to that of brain (Fig. 4D).

Total radioactivity in the 8-h urine and feces was 29% and 18%, respectively, of the injected dose. By 24 h, we could account for 40% and 34% of the injected activity, respectively; thus only 26% appeared to remain in the body by 24 h.

PDMP metabolites

Liver samples (100 mg) from uninjected and experimental mice, taken 1 h after PDMP injection, were extracted as above. A known amount of [^{3}H]PDMP was added to one extract from the controls. The extracts were evaporated to dryness, partitioned, and counted as above. In these tissue extracts, it was necessary to include 0.88% KCl in the partition to prevent emulsion formation. All added counts were recovered but the lower layer from the injected animal contained only 16% of the initial activity and the upper layer contained 84% of the activity. A radioautogram of the total liver and kidney extracts (C-M-W 50:40:10) showed the presence of unmetabolized PDMP, as well as three well-separated, slower-migrating metabolites. The intermediate product contained 50–56% of the total radioactivity in the metabolites. It was thus evident that considerable metabolic conversion of PDMP, presumably oxidation followed by derivatization, had occurred in just 1 h.

By contrast, the upper layers from brain and spleen extracts 1 h after injection showed no radioactivity. It would appear that the metabolites were formed in the liver and kidney and rapidly excreted.

Efforts were made to identify the polar products in urine as a glucuronide or a sulfate ester, using glucuronidase (from *E. coli*, type VIII-A, Sigma Chemical Co.) and sulfatase from *A. aerogenes*, Sigma Type VI). The resultant material was analyzed by TLC as above and only a small portion of the polar metabolites was found to be hydrolyzed in the case of the former enzyme. It is not clear whether the metabolites could not be attacked by these enzymes or whether they were products of a novel pathway.

It was apparent from TLC analysis of urinary tritium that nearly all of the radioactivity in the urine was due to polar PDMP metabolites that partition into the water-rich partition system.

Body water

Dehydrogenation of labeled PDMP at the hydroxylic carbon, possibly by an alcohol dehydrogenase, would lead to labeled water and the ketonic analog of PDMP (the propiophenone derivative used to synthesize PDMP). The ketone has previously been found to inhibit ceramide glucosyltransferase by covalent combination in vivo (17). Body water was isolated by lyophilizing groups of five carcasses (after removing the organs to be studied further) for 48 h and collecting the condensate. The observed specific activities of the water were 63, 45, 66, 97, and 81 cpm/ml of water for the five time periods, 20 min, 1 h, 3 h, 8 h, and 24 h, respectively. Assuming that a typical mouse contained 60% water, or ~21 ml, the total activities correspond to roughly 0.9% of the injected PDMP activity.

Attack by cytochrome P450

[^{3}H]PDMP (1000 cpm), dried from solution in a tube, was incubated with a microsomal preparation from 50 mg of liver, together with 50 mM Tris-Cl$^-$ pH 7.4, 5 mM $MgCl_2$, and 1 mM $NADH^+$ in a total volume of 0.75 ml. Some tubes also contained 1 mM PB. The contents of the tube were sonicated in an ice bath with a dipping probe for 30 sec and incubated 30 min at 37°C. The lipids were extracted and partitioned, and the lipids in the lower layer were separated by TLC as above.

The tritium from the control tube incubated with boiled microsomes and from the tube containing PB appeared only in the PDMP spot, showing that the amine was stable under these conditions. The lipids from the tube lacking PB showed a spot slower moving than the PDMP spot, which contained 20% of the tritium in the sample. From these results it appeared that a microsomal mixed function oxidase was responsible for the rapid metabolism of PDMP in vivo and we accordingly examined PDMP metabolism in mice treated with the oxidase inhibitor.

Effect of piperonyl butoxide on PDMP metabolism

PB injection into the mice (Study 2) produced no noticeable behavior response but, after PDMP injection, body movements were seen at intervals in the mice pretreated with the butoxide. These consisted of leg extensions, tail movements, head movements, and difficulty in walking. The spasms decreased in intensity after 8 h. They resembled those seen in untreated mice that were injected with higher doses of PDMP and could be attributed to higher levels of PDMP in the pretreated mice.

At various time intervals, the major organs were extracted with 24 vol of hexane–isopropanol. The tritium levels in the total extracts were much higher in the PB-treated mice, showing that blocking PDMP oxidation led

to much higher net activity and prolonged retention. In the case of extracts taken from PB mice, liquid/liquid partitioning showed that the lower layer (where PDMP is to be found) contained all of the radioactivity from brain and spleen and ~90% of the tritium from liver and kidney (although the 24-h mice had a higher proportion in the upper layer). In the extracts from the mice that had not been pretreated with PB, ~50% of the tritium at 20 min and ~20% at 1 h appeared in the lower layer. Thus, we saw again the very rapid oxidative metabolism in normal mice.

For a more precise picture of the effects, we isolated the PDMP by TLC from the lipid extracts, as before. It is evident from **Fig. 5** that the level of PDMP was much higher in all four organs of mice treated with PB. The untreated animals lost substantially all their PDMP within 4 h while the PB-treated animals had substantial concentrations of PDMP for >16 h. The maximally achieved concentrations at the early time points were also much higher in the PB mice. At 16 h, the concentration of PDMP in all organs was ~50 μM, far more than the concentration needed to block GlcCer synthesis in vitro.

A striking difference was seen in the PDMP levels in kidney (Fig. 5A), which were significantly higher than in the other organs. This suggests that kidney contains some organ-specific substance with affinity for the drug. This higher binding may explain the marked decreases in kidney weight and GlcCer content often seen in PB/PDMP-treated mice (9). In a previous study too we had found that repeated doses of PDMP, even without PB, specifically produced kidney shrinkage (4).

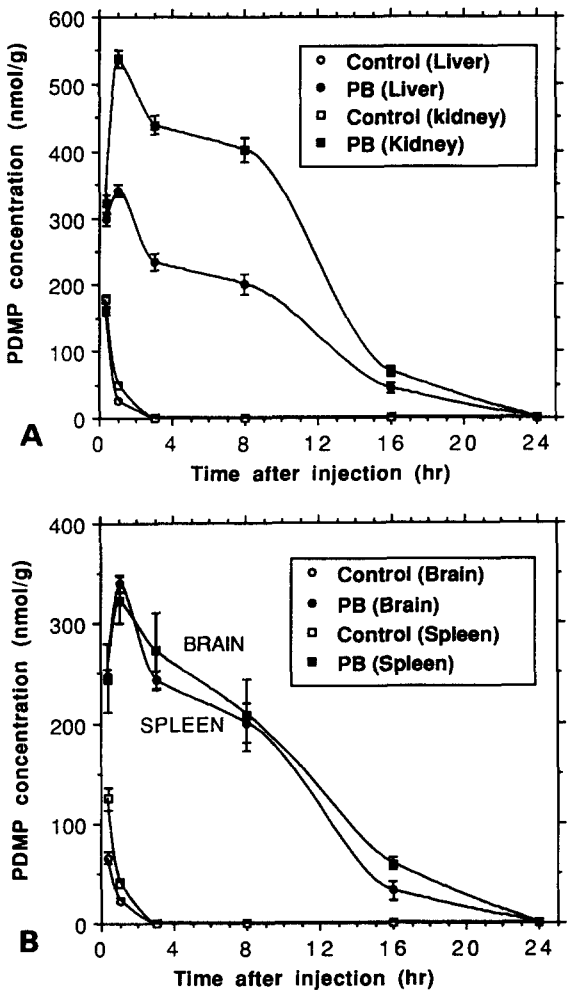


Fig. 5. Effect of pretreatment with piperonyl butoxide on the level of PDMP. 5A: Liver and kidney. 5B: Brain and spleen. The lower curves are from mice injected with PDMP, the upper curves are from mice injected with both PDMP and piperonyl butoxide.

Effect of PB and cimetidine on PDMP oxidation

The synergistic effect of PB was confirmed using intramuscular injection of the PB and subcutaneous injection of the PDMP (Study 3, **Table 1**). Cimetidine, another inhibitor of mixed function oxidase (18, 19), was injected intramuscularly. At the lower dosage of PDMP that was used here (40 mg/kg), no initial adverse symptoms were seen even in the PB-treated mice. Analysis of four organs for PDMP content revealed the previously observed rapid disappearance of the inhibitor from the organs of control mice (line 1, Table 1). No data are shown for the 3-h and 16-h mice as no PDMP could be detected. Prior treatment with PB produced marked elevation and prolongation of

TABLE 1. Effects of piperonyl butoxide (600 mg/kg) and of cimetidine (200 mg/kg) on the rate of metabolic degradation of D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (40 mg/kg)

Treatment	Liver	Kidney	Brain	Spleen
Controls - 1 h	10 ± 1.3	19.4 ± 1.9	9.0 ± 1.0	16 ± 1.6
PB[a] – 1 h	126 ± 3.4	205 ± 4.6	126 ± 3.2	119 ± 9.1
PB[a] – 3 h	94 ± 5.0	175 ± 7.5	97 ± 3.9	107 ± 20.1
PB[a] – 16 h	12 ± 3.1	22 ± 3.1	10 ± 4.2	18 ± 2.2
Cimetidine[b] – 1 h	87 ± 1.1	138 ± 1.9	84 ± 1.1	78 ± 3.8
Cimetidine[b] – 3 h	42 ± 1.3	73 ± 2.0	48 ± 1.3	54 ± 5.9

Values are the concentrations of PDMP in nmol/g ± SD.
[a]Injected 4 h before the PDMP.
[b]Injected 30 min before the PDMP.

all tissue levels of PDMP, the level being similar at 16 h to that in the 1-h control mice. Cimetidine, a commonly used antagonist of histamine H_2 receptors in human therapy, for reducing stomach acid formation, also showed distinct protective action, albeit less effectively than PB in the test paradigm used here. No PDMP could be detected in the 16-h mice. As noted before, PDMP concentrations were markedly higher in kidney.

Chemical and enzyme changes after PDMP and PDMP-PB injection (Study 4).

Comparison of the control and PB-control mice showed no differences. However, a significant decrease in kidney size was seen 24 h after PDMP injection (– 23%) and 8 h after PDMP/PB injection (– 11%). The body weights were not significantly lower.

Glucosyltransferase. There was no significant difference in the specific activities of glucosyltransferase assayed in liver, kidney, and brain in the two groups of control mice (Myrj-saline or PB-Myrj-saline) killed at 8 h. However, the kidneys of animals injected with PDMP, without PB, showed a distinct decrease in specific activity in the 3-h and 8-h mice (**Fig. 6**, upper dashed line). This decrease was not due to residual PDMP in the homogenized tissue, which was diluted 50-fold for the assay, since it was unaffected by diluting the homogenate further. (While there is the possibility that PDMP was irreversibly bound to the transferase, in vitro assays had shown that it was a reversible inhibitor.)

The effect of PDMP was clearly enhanced by prior injection with PB (Fig. 6, lower dashed line). The maximum decrease in activity was 45% at 3 h. Synthase activity after 24 h was 11% (significantly) higher than the control activity, apparently an overinduction of the enzyme.

Liver also showed a rapid drop in synthase activity (**Fig. 7**), significantly different from the controls in the 3-h (PDMP alone) and 3- and 8-h points (PDMP + PB). Here too PB markedly increased the effectiveness of PDMP; the maximum depression in activity was 38%.

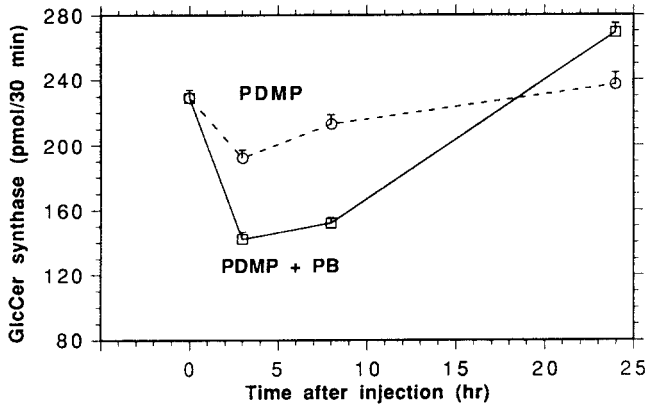


Fig. 7. Effect of a single injection of D-PDMP with or without a prior injection of piperonyl butoxide as in Fig. 6. The dashed line shows the synthase activity, measured in 2 mg of liver, of animals injected only with PDMP. The solid line is derived from mice injected with both inhibitors.

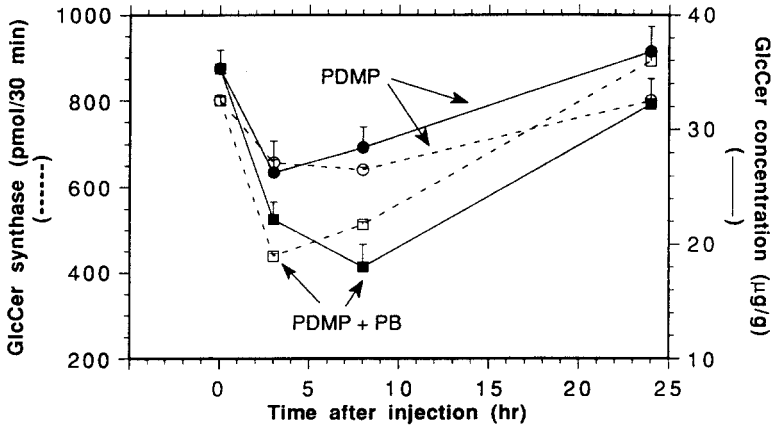
The over-induction phenomenon was visible in liver too, at the 24-h point.

The synthase activity in brain was not significantly affected except in the 8-h combined drug-treated mice (– 17%).

Lipid analyses. The GlcCer content of kidney was significantly decreased by both drug treatments at the 3- and 8-h time points (Fig. 6, solid lines). As with the synthase measurements, PB clearly enhanced the effect of PDMP; the maximum decrease in GlcCer level was 50% at 8 h.

The level of ceramide, the precursor of GlcCer, was significantly elevated in kidney by PDMP treatment, especially with PB (**Fig. 8**). We have seen this effect before in treated cells (7). Evidently ceramide synthesis continues normally despite the reduction in glucosylation. The level of ceramide dihexoside (GalGalCer and GalGluCer) was significantly low only in the 8-h PDMP-PB mice. The maximal depression was 17%. Apparently synthesis by galactosylation of residual GlcCer and by hydrolysis of the higher GSLs was almost sufficient to maintain the level of the dihexoside.

Fig. 6. Effect of a single i.p. injection of D-PDMP (80 mg/kg), with or without a prior i.p. injection of piperonyl butoxide (600 mg/kg). The dashed lines show the synthase activity, measured in duplicate 4-mg portions of kidney, and are derived from five pairs of kidneys for each point. The solid lines show the concentration of GlcCer, each value being the mean of ten TLC analyses (five mice analyzed in duplicate). The error bars represent one SD (they may be too small to see in the case of the enzyme activities). The control values, actually obtained from mice killed 8 h after the saline-Myrj injection, have been placed at the zero time. The data for GlcCer content are based only on the least polar monohexosides, containing nonhydroxy fatty acids and sphingosine. The more polar monohexoside TLC bands changed in intensity in parallel with the values shown here.



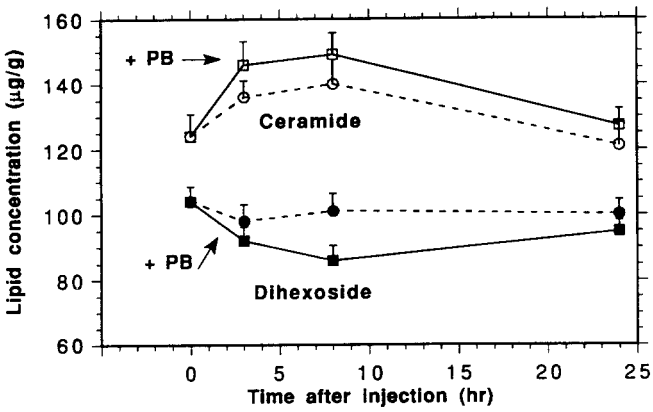


Fig. 8. Changes in ceramide and dihexoside levels of kidney after injecting PDMP ± piperonyl butoxide. The dashed lines refer to animals with PDMP alone. Only the two upper bands of ceramide dihexoside (containing nonhydroxy fatty acids and sphingosine) were analyzed. The more polar dihexosides showed visible changes similar to those seen for the less polar dihexosides.

Analysis for sphingomyelin content showed no statistically significant changes, although the 3-h and 8-h mice treated with PB and PDMP were 9% and 12% higher, respectively. These increases are reasonable in view of the increases seen in ceramide, the precursor of sphingomyelin.

DISCUSSION

Our finding that PDMP is rapidly metabolized and excreted explains why it was necessary to use such high dosage levels in vivo to achieve anticancer effects (4). From the excretion curves, it appears now that most of the effect was achieved in the first few hours after injection. This might seem like too little time to develop a deficiency in GSL content of the tumor cells, but it is possible that these cells have a high turnover rate for these lipids. In several kinds of cancer, GSLs appear in the blood at a relatively high concentration, apparently due to a shed ding phenomenon (20–22). Thus the turnover rate of GSLs in tumor cells is not simply due to synthesis and hydrolysis.

Even in normal mice, we have found that a single injection of PDMP produced a 35% decrease in kidney GlcCer within 5 h (9). The higher GSLs do not decrease as rapidly as GlcCer because they continue to be synthesized from the available GlcCer for a while. This was seen also in Fig. 8. The recycling phenomenon was recently demonstrated by direct means (23). Thus, one could hypothesize that the anticancer and kidney-shrinking effects of PDMP are due primarily to the decrease in GlcCer rather than to decreases in the more complex GSLs. It may be recalled that patients with the genetic disorder, Gaucher's disease, suffer from an accumulation of GlcCer in liver and spleen and that both organs grow excessively. Moreover, the growth of Ehrlich ascites cells in mice was accelerated by intraperitoneal contact with GlcCer (4).

PDMP apparently acts to inhibit GlcCer synthase by two mechanisms: one is by direct, reversible contact with the enzyme, as shown by in vitro assays, and the other is by oxidation, which we found in this study by counting the body water. The oxidation process most likely produces the ketone form of PDMP, which binds to the synthase irreversibly (17). The former effect does not show up when the organs of injected mice are assayed, if the tissue concentration in the assay tube is kept low, but the second effect is detectable at any tissue concentration. Mice given a single injection of PDMP and killed at various time intervals showed significant decreases in synthase activity of liver and kidney after a delay of a few hours; the effect gradually disappeared as new enzyme molecules were synthesized (9).

Our finding that PDMP degradation could be efficiently blocked by blocking microsomal monooxygenase with PB makes it possible to greatly prolong the action of the drug and hasten the disappearance of tissue GSLs. While our present study has not identified the structures of the polar metabolites formed, it is likely that they are the results of an initial oxidative attack by some enzyme system that can be inhibited by two structurally different drugs known to inhibit P450 systems. Further characterization of the system will require work with larger animals (in order to isolate the products) and with additional inhibitors known to block specific P450 isozymes. We are now testing the efficacy of the mixture of inhibitors and have found a remarkable ability to shrink the ascitic fluid produced in nude mice by inoculation with human colon cancer cells (G. S. Shukla and A. Shukla, unpublished results).

Piperonyl butoxide was developed as a synergist for use with pyrethrins in insecticides (24). Apparently its effectiveness is due to its ability to block insect monooxygenases that normally inactivate pyrethrins. The advantage of the synergist is that it greatly decreases the need for the active drug, making insecticides much less expensive. It would seem a useful extrapolation to adapt this inhibitor (or one designed to act more effectively in humans) for use with therapeutically useful drugs that are quickly eliminated from the body by P450 action. Not only would such a synergist reduce the cost of drugs—no trivial problem—but it could also reduce the variability in patients who respond differently to a standard drug dose because of individual differences in their drug-specific P-450s. In addition, it is becoming increasingly clear that the problem of drug interactions is due in some cases to the anti-monooxygenase effect of one of the drugs; this effect would be eliminated as a factor if one could deliberately block these enzymes. This factor is nicely illustrated by a recent demonstration that antimalarial drugs affect other

drugs this way (25). Of course it would be necessary to reevaluate the metabolism of each therapeutic drug under these conditions.

Piperonyl butoxide has been reported to be remarkably nontoxic despite the need for normal functioning of monooxygenases. At the level of 1% of the diet, it was tolerated well by rats over a 16-week period (26). Treatment of rats, cats, rabbits, and dogs on the eye surface, skin surface, and stomach yielded the conclusion that it was not markedly toxic (27).

A recent report has described similar sparing effects on an important drug, retinoic acid, by administration of a different P-450 inhibitor, ketoconazole, that is normally used therapeutically as a fungicide (28). This report, like ours, emphasizes the potential therapeutic value of using synergistic P-450 inhibitors.

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