

Induction of liver microsomal cytochrome P-450 2B1 by dimethyl diphenyl bicarboxylate in rats¹

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ABSTRACT Dimethyl diphenyl bicarboxylate (dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxy-diphenyl-2,2'-bicarboxylate, DDB), a synthetic mimic of the natural product schizandrin C, is used in China as a hepatoprotective agent to improve the liver functions of patients with hepatitis or under cancer chemotherapy. In this study, we investigated the effects of DDB on liver microsomal drug-metabolizing enzymes. When male Sprague-Dawley rats were treated with a daily intragastric dose of DDB (200 mg · kg⁻¹) for 3 d, the microsomal pentoxyresorufin dealkylase activity and P-450 2B1 protein levels were markedly increased. The fold increase was lower than that by phenobarbital (75 mg · kg⁻¹, ip once daily × 3 d). The level of P-450 2B1 mRNA was elevated by DDB but the magnitude of the elevation was much less than that caused by phenobarbital. DDB also increased the rates of testosterone hydroxylation at positions 16β, 16α, 6β, and 2β as well as the rate of ethoxyresorufin dealkylation, suggesting moderate increases in the levels of P-450 3A and P-450 1A1 in addition to the huge increase in P-450 2B1. The level of glutathione S-transferase was also slightly increased, but the levels of P-450 2E1 and NAD(P)H: quinone oxidoreductase were not changed. The results indicate that DDB is an inducer of P-450 2B1.

KEY WORDS dimethyl diphenyl bicarboxylate; liver microsomes; cytochrome P-450; testosterone;

cytosol; glutathione transferases; NADPH-ferrihemoprotein reductase

Dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxy-biphenyl-2,2'-dicarboxylate (dimethyl diphenyl bicarboxylate, DDB) is a synthetic mimic of the natural product schizandrin C, isolated from the commonly used traditional Chinese medicinal plant, *Fructus Schizandrae*⁽¹⁾. DDB can protect against liver injuries caused by CCl₄, D-galactosamine, thioacetamide, and prednisolone in rodents^(2,3). In clinical studies, DDB was effective in lowering serum glutamic-pyruvic transaminase (SGPT) levels, and in improving liver functions in patients with chronic hepatitis or receiving cancer chemotherapy with methotrexate and 5-fluorouracil. The induction of hepatic microsomal drug-metabolizing enzymes in rats by DDB and schizandrin C has been reported⁽⁴⁾. Their results indicated that cytochrome P-450 (P-450) species induced by DDB showed the same chromatographic and immunochemical behavior as phenobarbital-induced P-450. Since P-450s are involved in the detoxification or activation of toxicants and carcinogens, the possibility that hepatoprotection of DDB may be related to its effect on P-450 isozymes is of considerable interest.

In the present study, the effects of DDB on different P-450 isozymes were analyzed by measuring the activities of pentoxyresorufin, ethoxyresorufin, and N-nitrosodimethylamine (NDMA) dealkylases as well as by

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immunoblot analysis. Testosterone metabolism was also used to assess changes in the composition of P-450 isozymes in the microsomes after DDB treatment. In addition, the induction of P-450 2B1&2 by DDB at the mRNA level was compared with that by phenobarbital. The effect of DDB on NAD(P)H: quinone oxidoreductase and glutathione *S*-transferase was also considered since these two enzymes play important roles in the detoxification of xenobiotics.

MATERIALS AND METHODS

Materials DDB was obtained from Institute of Materia Medica, Chinese Academy of Sciences (Beijing, China). Ethoxyresorufin and 7-pentoxoresorufin were obtained from Pierce Chemical Co (Rockford IL). Glutathione, NDMA, and 1,2-dichloro-4-nitrobenzene (DCNB) were purchased from Aldrich Chemical Co (Milwaukee WI). Testosterone and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sigma Chemical Co (St Louis MO). Testosterone metabolite standards and antibodies against P-450 2B1 were kindly provided by Drs Paul E THOMAS and Zaid JAYYOSI of Rutgers University.

Rats and microsomes Sprague-Dawley ♂ rats (50–70 g initial body weight) were obtained from Taconic Farms, Inc (Germantown NY). They were kept in air-conditioned quarters with a 12-h light-dark cycle, and given a commercial laboratory chow (Ralston Purina Co, St Louis MO) and water *ad lib*. DDB was administered intragastrically at a daily dose of 200 mg · kg⁻¹ in 2% Tween-80 for 3 d. Phenobarbital was given by three daily intraperitoneal injections of 75 mg · kg⁻¹ in saline per day. Rats in control groups received either saline or 2% Tween-80 in saline. Liver microsomes were prepared by differential centrifugation and washed once with a solution containing KCl 154 mmol · L⁻¹ and EDTA 10 mmol · L⁻¹ (5). The microsomes were stored frozen in small portions at -70°C.

Enzyme assays Protein and P-450 were determined as described previously (6). NADPH-

cytochrome P-450 reductase activity was assayed using cytochrome *c* as an artificial electron acceptor. NDMA demethylase was determined by a colorimetric method based on the Nash reaction (7,8). *O*-Dealkylation of ethoxyresorufin and pentoxoresorufin was assayed as described by Lubet *et al* (9) using a Perkin-Elmer model 512 double beam fluorescence spectrophotometer with the excitation wavelength set at 522 nm, the emission wavelength set at 586 nm, and the entrance and exit slits set at 10 nm. NAD(P)H: quinone oxidoreductase was assayed with NADH as the electron donor, menadione as the electron acceptor, and in the presence of Triton-100 as the activator. The assay medium consisted of Tris-HCl buffer 50 mmol · L⁻¹ (pH 7.5), NADP 1 mmol · L⁻¹, 0.8% Triton-100, and cytochrome *c* 0.5 mmol · L⁻¹. The spectrophotometric determination of glutathione *S*-transferase activity was carried out in the presence of potassium phosphate 100 mmol · L⁻¹ (pH 6.5) with glutathione 1 mmol · L⁻¹ and CDNB 1 mmol · L⁻¹, or with glutathione 5 mmol · L⁻¹ and DCNB 1 mmol · L⁻¹ (10). Gel electrophoresis and immunoblot analyses were performed (7). Densitometry was conducted using a Shimadzu CS-930 TLC Scanner (Shimadzu Corp, Kyoto, Japan).

Slot blot analysis Cytochrome P-450 2B1 cDNA which was cloned into the *EcoRI* site of pUC18 was obtained from Dr Frank J GONZALEZ (National Cancer Institute, Bethesda MD). The DNA was digested with *EcoRI* and the 1.8 kb cDNA fragment was recovered using DEAE cellulose paper (NA 45, Schleicher and Schuell, Keene NH) after electrophoretic separation on a 1% agarose gel. The fragment was made radioactive by nick-translation using [α -³²P]dCTP (11). Total RNA was isolated from rat liver using the guanidinium thiocyanate-phenol-chloroform extraction method (12). RNA was quantified by measuring the absorbance at 260 nm (one A₂₆₀ unit = 40 μ g RNA · ml⁻¹). Total RNA from liver samples were applied onto nitrocellulose filters using a slot blot apparatus (Minifold II; Schleicher and Schuell). The amounts of the samples applied varied from 0.5 to 6 μ g per slot.

Testosterone hydroxylation assay Testosterone metabolism was assayed by a modification of the method of Sonderfan *et al*⁽¹³⁾. In brief, the assay mixture contained Tris buffer $50 \text{ mmol} \cdot \text{L}^{-1}$ (pH 7.4), MgCl_2 $10 \text{ mmol} \cdot \text{L}^{-1}$, KCl $150 \text{ mmol} \cdot \text{L}^{-1}$, NADP $0.4 \text{ mmol} \cdot \text{L}^{-1}$, glucose 6-phosphate $10 \text{ mmol} \cdot \text{L}^{-1}$, glucose-6-phosphate dehydrogenase 0.2 units, microsomes 0.5 mg , and testosterone $250 \text{ nmol} \cdot \text{L}^{-1}$ in a total volume of 0.5 ml . Blank and standards were prepared in the absence of the NADPH -generating system. Reaction was started by the addition of the NADPH -generating system and stopped by adding 2 ml dichloromethane. All samples were vigorously mixed on a vortex device for 2 min . After two phases were separated by low speed centrifugation ($3000 \times g$ for 2 min), 0.5 ml of the organic phase was transferred to a microcentrifuge tube and evaporated under vacuum. The residue was redissolved in $100 \mu\text{l}$ HPLC solvent A (tetrahydrofuran : water, $10 : 90$), and a $50 \mu\text{l}$ aliquot was injected onto a Supelco C_{18} $5 \mu\text{m}$ HPLC column ($15 \times 0.46 \text{ cm}$). The following solvent program was used at a flow rate of $1.5 \text{ ml} \cdot \text{min}^{-1}$: the column was eluted with solvent A for the first 22 min ; from 22 to 48 min solvent A was mixed with solvent B (tetrahydrofuran : methanol : water, $5 : 70 : 25$) in a linear gradient to reach 30% A and 70% B, followed by a linear gradient to reach 100% B from 48 to 52 min ; and from 52 to 59 min 100% A was obtained in a linear gradient. The Waters 840 HPLC system included dual model 512 pumps, a model 490E detector set at 254 nm , and model 380 Digital computer, and a model 712 WISP autoinjector. The concentration of each metabolite was determined by comparing its integrated area to that of the corresponding standard compound (2.4 nmol each) that was subjected to a similar incubation and extraction. The recovery for the standards was $>95\%$.

RESULTS

The effects of DDB and phenobarbital on the induction of various monooxygenase activities are shown in Tab 1A. DDB treatment,

once daily $\times 3 \text{ d}$, significantly increased the microsomal content of total P-450. The activity of microsomal NADPH -cytochrome *c* reductase which reflects the activity of NADPH -P-450 reductase was also increased by 33% due to DDB treatment. These trends are in agreement with the previous results by Liu *et al*⁽⁴⁾. In order to study the specific P-450 isozymes that are affected, pentoxyresorufin, ethoxyresorufin, and NDMA were used as substrates for enzyme assays. The dealkylation of pentoxyresorufin, which is highly selective for P-450 2B1, was increased 200- and 280-fold by DDB and phenobarbital, respectively. In contrast, dealkylation of ethoxyresorufin, which mainly reflects P-450 1A1 activity was only increased 2.0- and 2.7-fold, respectively. The demethylation of low concentration of NDMA, indicative of the level of P-450 2E1, was unaffected by both DDB and phenobarbital.

Since these results suggest that DDB caused increases in microsomal enzyme activities similar to those by phenobarbital, the microsomal samples were examined using anti-2B1 IgG (which also recognizes P-450 2B2) in immunoblot analysis (Fig 1). In the microsomes from untreated rats, the band is primarily due to P-450 2B2 (M_r $52\,500$)⁽¹⁴⁾. Treatment of rats with DDB and phenobarbital induced a protein (the lower band) which had the same electrophoretic mobility as P-450 2B1 (M_r $52\,000$). Since P-450 2B1 and P-450 2B2 bands could not be accurately quantified separately, they were quantified together as P-450 2B1&2. The data Tab 1B indicate that the amounts of immunodetectable P-450 2B1&2 (mainly P-450 2B1) were 28- and 89-fold higher in DDB- and phenobarbital-induced microsomes, respectively, than that (mainly P-450 2B2) in control microsomes. Phenobarbital treatment

Tab 1. Effects of DDB and phenobarbital on rat liver microsomes. $n=5$ microsomal preparations. $\bar{x} \pm s$.
* $P > 0.05$, ** $P < 0.05$, *** $P < 0.01$ vs Control.

	Control	DDB	Phenobarbital
A) Total P-450 (nmol · mg ⁻¹)	0.90 ± 0.03	1.45 ± 0.20***	2.13 ± 0.14***
Dealkylation: Reductase (nmol · min ⁻¹ · mg ⁻¹)	228 ± 33	303 ± 31***	353 ± 22***
Pentoxoresorufin (pmol resorufin · min ⁻¹ · mg ⁻¹)	2.5 ± 0.8	519 ± 202***	698 ± 220***
Ethoxoresorufin (pmol resorufin · min ⁻¹ · mg ⁻¹)	40.1 ± 3.2	79 ± 13***	107 ± 6***
NDMA (nmol HCHO · min ⁻¹ · mg ⁻¹)	1.29 ± 0.24	1.25 ± 0.60*	1.14 ± 0.21*
B) Cytochrome P-450 2B1&2 protein ^(a)	2.4 ± 0.7	66 ± 22***	215 ± 50***
Cytochrome P-450 2B1&2 mRNA ^(a)	5.2 ± 2.1	66 ± 43***	640 ± 104***
C) Testosterone metabolism (nmol metabolite · min ⁻¹ / mg protein)			
7 α & 15 β	0.69 ± 0.08	0.68 ± 0.06*	1.13 ± 0.10**
6 β	0.85 ± 0.16	1.35 ± 0.10***	2.1 ± 0.5***
16 α	0.09 ± 0.03	0.54 ± 0.09**	0.75 ± 0.17***
16 β	0.01 ± 0.01	0.29 ± 0.05***	0.42 ± 0.15***
2 β	0.14 ± 0.02	0.24 ± 0.02**	0.49 ± 0.09***
Androstenediones	0.14 ± 0.02	0.61 ± 0.07***	0.64 ± 0.18***
D) Cytosolic enzymes (nmol · min ⁻¹ / mg protein)			
Glutathione S-transferase: DCNB	20.6 ± 4.6	31.2 ± 5.4**	33.4 ± 3.4***
CDNB	506 ± 87	660 ± 83**	950 ± 156***
NAD(P)H-quinone oxidoreductase	1 170 ± 407	976 ± 265*	1 476 ± 411*

^(a) Arbitrary units for the peak areas of immunostained bands (protein) and autoradiographed slots (mRNA) as determined by densitometry.

is known to increase the levels of several mRNA species, including the one coding for 2B1⁽¹⁵⁾. In order to evaluate whether DDB has the same effect as phenobarbital, liver 2B1 mRNA levels were determined by slot blot analysis using a cDNA probe (which cross-hybridizes with P-450 2B2 mRNA). Treatment of rats with phenobarbital caused more than 100-fold induction of P-450 2B1&2 mRNA, whereas DDB produced a 13-fold increase (Tab 1B). Similar results were obtained in another set experiment.

Hydroxylation of testosterone has been shown to be catalyzed by different P-450 isozymes regioselectively and the profile of metabolites reflects the composition of P-450 isozymes in microsomes⁽¹⁴⁾. In rat liver, the 16 β -hydroxylation of testosterone is mainly catalyzed by P-450 2B1, and to a much lesser extent by P-450 2B2. Large increases in 16 β -hydroxytestosterone formation (29- and



Fig 1. Immunoblot analysis of hepatic microsomes from control, DDB-induced, and phenobarbital-induced rats. Microsomes were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis using anti-P-450 2B1 IgG. Individual microsomal preparations were obtained from control (lanes a-c, 20 μ g protein/lane) or from rats treated with DDB (d-h, 1.25 μ g protein/lane) or phenobarbital (i-m, 1.25 μ g protein/lane). Lanes n and o contained the mixture (8.6 μ g) of control and phenobarbital-induced microsomes (0.67 μ g), respectively. The relative densities of major bands in the lanes are indicated in Tab 2.

42-fold) after treatments with DDB and phenobarbital, respectively (Tab 1C), reflect the induction of P-450 2B1. The 16 α -hydroxylation is catalyzed by P-450 2B1, P-450 2C11 and perhaps other P-450 form, and was affected to smaller extents (6- and 8-fold increase) by the treatments with DDB and phenobarbital, respectively. In addition, DDB treatment increased the 6 β - and 2 β -hydroxylation of testosterone by 60-70%, possibly a consequence of the induction of P-450 3A. Testosterone 17-oxidation leading to the formation of androstenedione was also increased 4-fold by DDB.

The glutathione *S*-transferase activity with two substrates, DCNB and CDNB, was increased significantly by DDB. Similar induction was also seen in phenobarbital-treated rats. No effect on NAD(P)H: quinone oxidoreductase activity was found in either DDB- or phenobarbital-treated rats (Tab 1D).

DISCUSSION

The present results demonstrate that the P-450 2B1-dependent pentoxyresorufin dealkylase activity was increased (200-fold) by DDB. This inductive effect is similar to that of phenobarbital and the result is in agreement with the observation of Liu *et al*⁽⁴⁾. Immunoblot analysis showed that a band corresponding to P-450 2B1 was markedly increased by DDB. Because of the low intensity of P-450 2B1 in control microsomes and because P-450 2B1 and P-450 2B2 were not quantified separately, the fold of P-450 2B1 induction could not be calculated accurately. It should be much higher than the value shown in Tab 1B, in which P-450 2B1 and P-450 2B2 were quantified together. DDB (600 mg · kg⁻¹, ig) was slightly less effective than phenobarbital (225 mg · kg⁻¹, ip) in the induction of P-450 2B1, according to protein

level and pentoxyresorufin dealkylase activity. However, at the mRNA level, DDB was a much weaker inducer than phenobarbital. The reasons for the disparity between these parameters are not known. DDB also caused moderate increases in P-450 1A1-dependent ethoxyresorufin dealkylation (2-fold) and 3A-dependent testosterone 6 β -hydroxylation (by 60%). But DDB did not affect P-450 2E1-dependent NDMA demethylase activity. The effects of phenobarbital treatment on these P-450 isozymes were similar to those of DDB.

Certain P-450 isozymes are known to be induced by their substrates. To determine whether DDB is a substrate for P-450 2B1 microsomal DDB metabolism was determined by measuring formaldehyde formation. With microsomes from untreated rats, the rate was 0.11 nmol · min⁻¹ / mg protein, possibly from the *O*-demethylation reaction. Treatment of rats with phenobarbital and DDB did not enhance the rate of the metabolism, suggesting that DDB is not a good substrate for P-450 2B1.

The presently observed induction of P-450 2B1 may contribute to the rate of metabolism of drugs and xenobiotics in animals and affect their toxicity. This mechanism of action may be related to the protective effect of DDB against the toxicity induced by cancer chemotherapeutic drugs such as methotrexate and 5-fluorouracil. A slight increase of cytosolic glutathione *S*-transferase activity with both substrates, DCNB and CDNB, was observed in liver of rat treated with DDB and phenobarbital. This result suggests that the induction of glutathione *S*-transferase by DDB may also contribute to the detoxification of certain xenobiotics.

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## Leukotriene C<sub>4</sub> receptors in cultured smooth muscle cells from bovine anterior cerebral arteries and microcerebrovasculatures<sup>1</sup>

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**ABSTRACT** Specific receptors for leukotriene C<sub>4</sub> (LTC<sub>4</sub>) were identified on smooth muscle cells isolated from bovine anterior cerebral arteries (BACASMC)

and bovine microcerebrovasculatures (BMSMC). [<sup>3</sup>H]LTC<sub>4</sub> specific bindings to both cells at a fixed input reached the maxima at 60 min and 20 min, respectively. With incremental inputs of radioligand and a constant cell number, [<sup>3</sup>H]LTC<sub>4</sub> specific bindings reached a plateau indicative of a saturable binding site. Analysis of Scatchard plots demonstrated a

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