Biochemical Pharmacology, Vol. 46, No. 5, pp. 933–943, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

METHOXYRESORUFIN AND BENZYLOXYRESORUFIN: SUBSTRATES PREFERENTIALLY METABOLIZED BY CYTOCHROMES P4501A2 AND 2B, RESPECTIVELY, IN THE RAT AND MOUSE

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(*Received* 26 *October* 1992; *accepted* 14 *April* 1993)

Abstract—The cytochrome P450 isozyme specificity for the *O*-dealkylation of methoxyresorufin (MTR) and benzyloxyresorufin (BZR) in the rat and mouse was investigated. The induction of various alkoxyresorufin *O*-dealkylation activities was measured in male F344/NCr rats exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 3,4,5,3′,4′,5′-hexachlorobiphenyl. MTR and ethoxyresorufin (ETR) *O*-dealkylation activities were induced 30- and 80-fold, respectively, in the liver. ETR *O*-dealkylation activity was induced > 250-fold in the kidney, whereas the metabolism of MTR was induced only 30-fold in this extrahepatic tissue. Phenacetin, a fairly specific CYP1A2 inhibitor, caused concentration-dependent competitive inhibition of MTR *O*-dealkylation ($k_i \sim 20\ \mu M$ at $0.5\ \mu M$ substrate) in hepatic microsomes from 3,4,5,3′,4′,5′-hexachlorobiphenyl-treated rats. The corresponding k_i for inhibition of ETR *O*-dealkylation by phenacetin was $\geq 333\ \mu M$ at a $0.5\ \mu M$ substrate concentration. A monoclonal antibody displaying inhibitory activity against rat CYP1A1 inhibited ETR *O*-dealkylation activity, whereas it failed to inhibit MTR *O*-dealkylation activity. In contrast, a monoclonal antibody reactive with both CYP1A1 and CYP1A2 inhibited both *O*-dealkylation activities to an equal extent. Similar experiments, employing phenacetin or specific monoclonal antibodies, yielded comparable results when performed with mouse microsomes. The maximal induction of MTR *O*-dealkylation activity in mice was > 100-fold. The P450 isozyme specificity of BZR *O*-dealkylation was also examined in both rats and mice. Pregnenolone-α-carbonitrile, a strong inducer of CYP3A, only weakly induced BZR *O*-dealkylation activity. In addition, a monoclonal antibody that specifically inhibits CYP2B caused inhibition of BZR metabolism in microsomes from phenobarbital- or dexamethasone-pretreated rats. In B6C3F1 mice exposed to dietary Aroclor 1254, significant induction of hepatic MTR *O*-dealkylation activity was observed at concentrations lower than those required for the induction of ETR or BZR *O*-dealkylation. In summary, it would appear that MTR is a relatively specific substrate for CYP1A2 activity in rodents, while BZR appears to be relatively specific for CYP2B.

The CYP1A family includes two genes, CYP1A1 and CYP1A2, which show a relatively high degree of nucleotide homology (~75% in rats). There are numerous interesting aspects of these two cytochromes. The induction of these CYP proteins appears to be coordinately regulated in the liver [1, 2]. In general, the same compounds induce both of these proteins and the two proteins are induced with similar kinetics. Although the gene for CYP1A1 contains various xenobiotic-responsive and drug-responsive elements in its 5′-flanking region which allow for the binding of the occupied *Ah* receptor, it appears that the CYP1A2 gene may lack a functional xenobiotic-responsive element [3–5]. Perhaps of the greatest potential interest is the fact that these enzymes metabolize a great variety of xenobiotics, including many known or suspected carcinogens. Interestingly, individual isozymes in this subfamily preferentially metabolize different classes of carcinogens. Thus, CYP1A1 catalyzes the oxidation of a variety of relatively planar compounds, such as the polycyclic hydrocarbons [6, 7], whereas CYP1A2 preferentially metabolizes (*N*-hydroxylates) a variety of aromatic amines [8–10], some of which are known human carcinogens (including 4-aminobiphenyl, 2-naphthylamine and benzidine).

One of the greatest limitations encountered in the quantification of CYP1A2 has been the lack of a specific catalytic endpoint with which this isozyme can be monitored. Previously, the two most sensitive and specific catalytic assays for this cytochrome have been the *O*-deethylation of phenacetin (determined at low phenacetin concentrations [11, 12]) and the *N*-hydroxylation of certain tryptophan pyrolysis products [8, 10]. Both of these methodologies are technically difficult, involving GC/MS or HPLC analysis, and do not allow the reactions to be monitored in real time. In addition to these two assays, the decarboxylation of uroporphyrinogen has also been demonstrated recently to be mediated by

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CYP1A2 in the mouse [13] and, therefore, has the potential for use in assaying this isozyme.

The present study was based upon the observations reported by Namkung *et al.* [14] that purified rat CYP1A2 readily metabolizes methoxyresorufin (MTR*) while metabolizing ethoxyresorufin (ETR) at a much lower rate. In contrast, it was found that CYP1A1 preferentially metabolizes ETR but not MTR. These results implied that the *O*-dealkylation of MTR may prove to be a relatively simple method for monitoring CYP1A2 activity in rats.

In contrast to the relative paucity of data dealing with MTR, the benzyloxy analogue has generated significantly more (albeit somewhat contradictory) data. In the paper in which the use of MTR was discussed [14], it was also implied, on the basis of the use of purified CYP proteins, that benzyloxyresorufin (BZR) is metabolized by a number of different inducible and constitutive isozymes of cytochrome P450 (including CYP1A1, CYP1A2, CYP2B1/2 and CYP3A1/2) in the rat. The latter finding that various CYP proteins can catalyze BZR *O*-dealkylation is in substantial agreement with the data reported by Burke *et al.* [15]. Most recently, Chen and Eaton [16] have proposed that BZR *O*-dealkylation is mediated principally, but not exclusively by CYP3A in the rat, implying that this activity may be a marker of this isozyme subfamily.

In the present experiments, we have attempted both to confirm these findings in the rats, employing a variety of endpoints, and to expand these results, utilizing mice pretreated with different prototype inducers. Briefly stated, we confirmed that the alkoxyresorufin assays are simple, sensitive and rather selective assays for monitoring specific CYP activities in these two rodent species. Thus, CYP1A2 appears to preferentially catalyze MTR *O*-dealkylation, CYP1A1 preferentially metabolizes ETR, and CYP2B1/2 preferentially catalyzes the *O*-dealkylation of both BZR and pentoxyresorufin (PTR).

MATERIALS AND METHODS

Chemicals. $NADP^+$, NADPH, 7,8-benzoflavone, phenobarbital (PB) and dicumarol were obtained from the Sigma Chemical Co. (St. Louis, MO). MTR, ETR, BZR and PTR were obtained from Molecular Probes, Inc. (Eugene, OR). Aroclor 1254 and 3,4,5,3′,4′,5′-hexachlorobiphenyl (HCB) were obtained from Analabs, Inc. (North Haven, CT). Resorufin was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Pregnenolone-α-carbonitrile (PCN) was obtained from the Upjohn Co. (Kalamazoo, MI). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from the NCI Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO). Nitrocellulose paper was obtained from Schleicher & Schuell, Inc. (Keene, NH). The pre-cast mini gels were obtained from Novex (San Diego, CA), and other chemicals used for western blotting were purchased from Bio-Rad (Richmond, CA).

Treatment of animals. Male F344/NCr rats and male B6C3F1 and NIH/Swiss mice were obtained from the Animal Production Area, FCRDC. At 7 weeks of age, male rats or mice were administered three daily i.p. doses of dexamethasone (DEX; 250 mg/kg body wt) or PCN (60 mg/kg body wt) and killed by CO_2 asphyxiation 24 hr after the last dose. Additional male rats or mice were fed PB (500 ppm in the diet) for a period of 7 days, and were killed on the last day of feeding. Additional groups of rats were fed HCB (8 ppm in the diet) for a period of 5 days and were killed 24 hr later. Other male F344/NCr rats were injected once i.p. with 25 μg TCDD/kg body wt, and were killed 5 days later. The treatment regimens described generally did not result in significant decreases in body weight gains occurring over the course of the experiments. However, the rats exposed to the relatively high dose level of DEX employed displayed an 18% decrease in body weight gain, compared to the controls (data not shown). Male B6C3F1 mice were exposed to dietary Aroclor 1254 (0–500 ppm for a period of 14 days) and were killed on the last day of feeding. NIH/Swiss mice were given a single i.p. injection of TCDD (16 μg/kg body wt) and were killed 7 days later. Livers were excised from the individual rats and mice, weighed, and homogenized in 0.15 M KCl/0.2 M sucrose (4 mL/g wet liver weight, 4°). Post-mitochondrial (S-9), microsomal and cytosolic subfractions were obtained by sequential 9,000 and 105,000 *g* centrifugations.

Alkoxyresorufin O-dealkylase assays. Alkoxyresorufin *O*-dealkylation activities were measured in hepatic S-9s exactly as previously described [17]. The substrate concentrations employed were 5 μM, and the substrates were dissolved in dimethyl sulfoxide (DMSO). Protein levels in S-9 and microsomal fractions were measured using fluorescamine [18] with bovine serum albumin as standard.

Chemical inhibition studies. 7,8-Benzoflavone or phenacetin, each dissolved in DMSO, was added to a mixture containing S-9 protein, substrate, and buffer (0.05 M Tris, pH 7.4, 0.025 M $MgCl_2$; 2 mL total volume). After a 2-min incubation at room temperature, the reactions were initiated by the addition of NADPH.

Monoclonal antibodies. MAb C-8 is specifically directed against rat CYP1A1 and has been described previously [19, 20]. MAb 1-7-1 was raised against rat CYP1A and has been shown to react with both CYP1A1 and CYP1A2 in a variety of animal species [21]. MAb 2-66-3 was raised against rat CYP2B [22].

Immunoinhibition studies. Monoclonal antibodies were combined with rat or mouse microsomal protein in various ratios and were incubated in 50 mM Tris–HCl (pH 7.5), final volume 300 μL, for 20 min at room temperature. Following this time period, 2.0 mL of the assay mixture for determining alkoxyresorufin *O*-dealkylation activities (0.05 M

* Abbreviations: BZR, benzyloxyresorufin; DEX, dexamethasone; ETR, ethoxyresorufin; HCB, 3,4,5,3′,4′,5′-hexachlorobiphenyl; MTR, methoxyresorufin; PB, phenobarbital; PCN, pregnenolone-α-carbonitrile; PTR, pentoxyresorufin; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

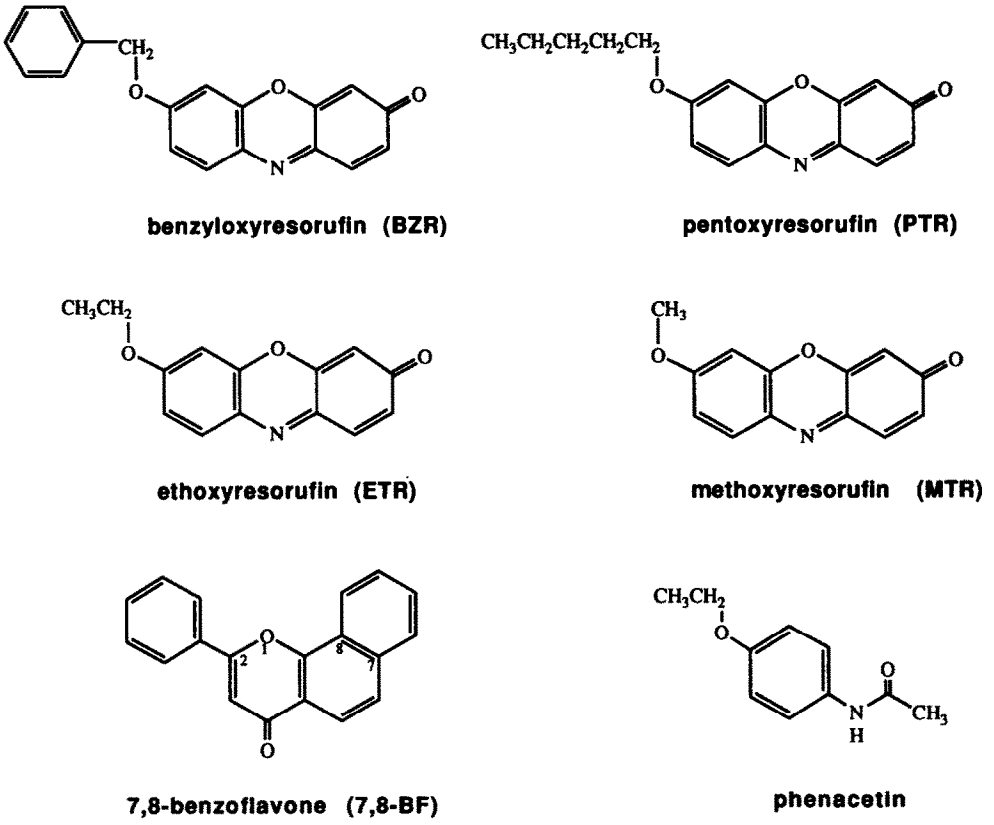

Fig. 1. Structural formulae of the P450 substrates and inhibitors employed in these studies.

Tris, 0.025 M $MgCl_2$, pH 7.4, containing 5 μM substrate) was added to the tubes, and the specific *O*-dealkylation activities were determined.

Immunodetection employing western blotting procedures. Protein content of liver microsomal samples was determined according to the standard procedure of Lowry *et al.* [23], using bovine serum albumin as the standard. Microsomes were diluted in sample buffer and loaded onto 8% pre-cast mini gels. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [24]. Proteins were then transferred to nitrocellulose according to Towbin *et al.* [25], at a constant voltage of 23 V for 1 hr. The nitrocellulose was rinsed, blocked for 2 hr in 5% nonfat dry milk, and probed with MAb 1-7-1. The blots were then probed with a secondary antibody (goat anti-mouse IgG, GIBCO-BRL, Gaithersburg, MD) conjugated to alkaline phosphatase, and bands were visualized by exposing the blots to color development solution (*p*-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt, in sodium bicarbonate buffer, pH 9.8).

RESULTS

The metabolism of a number of phenoxazone ethers (methoxy-, ethoxy-, pentoxy- and benzyloxyresorufin, Fig. 1) by S-9 samples prepared from the livers and kidneys of rats and mice treated with a variety of xenobiotics was studied (Tables 1 and 2). MTR *O*-dealkylation activity was highly induced in the livers of B6C3F1 mice treated with Aroclor 1254, Swiss mice treated with TCDD, and F344/NCr rats treated with HCB or TCDD. The metabolism of this substrate was more highly induced in the livers of mice (up to 172-fold) than in rats (20- to 26-fold), and was also induced 30-fold in the kidneys of rats treated with these xenobiotics, but was less strongly (11-fold) induced in the kidneys of mice. ETR metabolism was induced 47- to 70-fold in rat liver and 103- to 136-fold in mouse liver. This activity was induced ~250-fold in the rat kidney, but only ~30-fold in the mouse kidney. In terms of actual substrate turnover rates achieved, the maximal rates of MTR metabolism in rat liver were 20–25% of the rates of ETR metabolism. In contrast, in the rat kidney the maximal MTR *O*-dealkylation rate was < 7% of the maximal ETR *O*-dealkylation rate observed.

This induction pattern was confirmed by western blot analyses employing a monoclonal antibody cross-reactive with both CYP1A1 and CYP1A2. Thus, both CYP1A1 and CYP1A2 proteins were highly induced in liver microsomes, whereas only a single immunoreactive protein (CYP1A1) was induced in the kidney (Fig. 2).

Inhibition of the *O*-dealkylation of MTR or ETR

Table 1. Induction of alkoxyresorufin *O*-dealkylation activities in F344/NCr rats

Xenobiotic*	Alkoxyresorufin *O*-dealkylation activity† (pmol resorufin formed/min/mg S-9 protein)			
	MTR	ETR	BZR	PTR
Liver				
Control	21 ± 2	36 ± 3	29 ± 4	14 ± 2
HCB	420 ± 47	1700 ± 190	93 ± 12	41 ± 4
TCDD	550 ± 48	2500 ± 200	110 ± 21	ND‡
DEX	43 ± 5	96 ± 12	390 ± 39	180 ± 27
PCN	37 ± 4	90 ± 10	150 ± 27	42 ± 4
PB	40 ± 5	110 ± 8	1200 ± 190	510 ± 46
Kidney				
Control	0.54	0.97	ND	ND
TCDD	16	260	ND	ND

* Abbreviations and dosage regimens used: HCB (3,4,5,3′,4′,5′-hexachlorobiphenyl) was administered at 8 ppm in the diet for 5 days; TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) was administered as a single i.p. injection of 25 μg/kg body weight; DEX (dexamethasone) was administered as three daily i.p. injections of 250 mg/kg body weight; PCN (pregnenolone-α-carbonitrile) was administered as three daily i.p. injections of 60 mg/kg body weight; PB (phenobarbital) was administered at 500 ppm in the diet for 7 days.

† Values are means ± SD for three rats/treatment. Results shown for kidney activities are for samples pooled from three animals. All values have been rounded to two significant figures.

‡ ND, not determined.

Table 2. Induction of alkoxyresorufin *O*-dealkylation activities in B6C3F1 and NIH/Swiss mice

Xenobiotic*	Alkoxyresorufin *O*-dealkylation activity (pmol resorufin formed/min/mg S-9 protein)			
	MTR	ETR	BZR	PTR
Liver				
Control†	13 ± 2	21 ± 4	7 ± 1.4	3.2 ± 1
Aroclor 1254†	2200 ± 88	2900 ± 140	590 ± 120	230 ± 63
TCDD‡	1800	2200	31	11
DEX†	37 ± 6	56 ± 9	390 ± 47	130 ± 21
PCN†	21 ± 5	43 ± 8	23 ± 7	10 ± 2
PB†	36 ± 6	71 ± 7	370 ± 31	130 ± 19
Kidney				
Control‡	0.95	1.10	ND§	ND
TCDD‡	10	31	ND	ND

* Abbreviations and dosage regimens used: Aroclor 1254 was administered at 500 ppm in the diet for 14 days; TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) was administered as a single i.p. injection of 16 μg/kg body weight; DEX (dexamethasone) was administered as three daily i.p. injections of 250 mg/kg body weight; PCN (pregnenolone-α-carbonitrile) was administered as three daily i.p. injections of 60 mg/kg body weight; PB (phenobarbital) was administered at 500 ppm in the diet for 7 days.

† Values are means ± SD for three B6C3F1 mice per treatment. Values have been rounded to two significant figures.

‡ Values are for samples pooled from three NIH/Swiss mice per treatment. Values have been rounded to two significant figures.

§ ND, not determined.

by various concentrations of phenacetin (a prototype CYP1A2 substrate [10, 11], Fig. 1) is displayed in Fig. 3. When plotted as a double-reciprocal (Lineweaver–Burk) plot (Fig. 4), the inhibition curves obtained were consistent with competitive inhibition. The k_i values for phenacetin at MTR concentrations of 5, 1.6 and 0.5 μM were 500, 100, and 20 μM, respectively, in the presence of rat S-9.

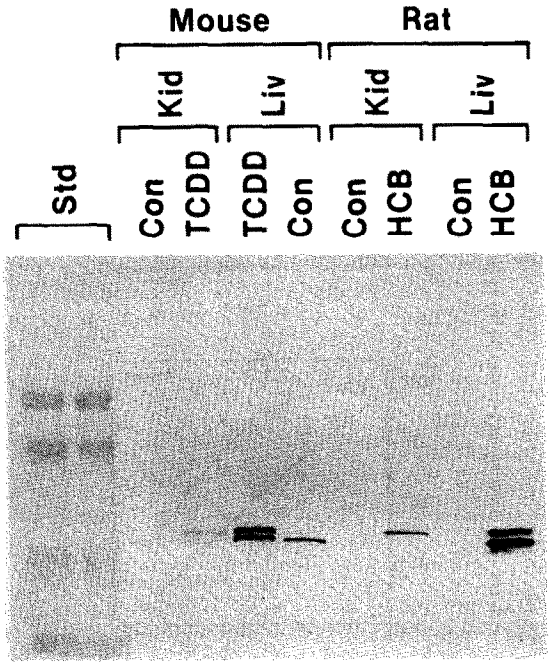

Fig. 2. Western blot, with MAb 1-7-1, of microsomal CYP1A protein from NIH/Swiss mice pretreated with TCDD and F344/NCr rats pretreated with HCB. The various lanes (from left to right) were loaded with molecular weight standards or microsomal protein as indicated: STD, molecular weight markers; mouse control kidney (20 μg protein loaded); mouse TCDD kidney (20 μg); mouse TCDD liver (0.5 μg); mouse control liver (10 μg); rat control kidney (20 μg); rat HCB kidney (20 μg); rat control liver (10 μg); rat HCB liver (1.0 μg).

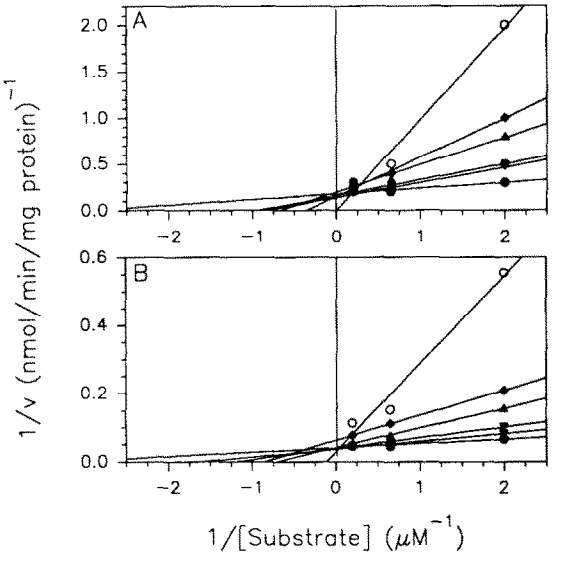

Fig. 4. Double-reciprocal plot of the inhibition, by phenacetin, of MTR *O*-dealkylation activity in S-9 from a HCB-treated rat (A) or a TCDD-treated mouse (B). The phenacetin concentrations employed were 0 (●), 5.5 (▼), 17 (■), 50 (▲), 150 (◆) and 450 (○) μM. Uninhibited MTR *O*-dealkylation activities were 420 and 1800 pmol/min/mg S-9 protein, respectively, in the rat and mouse.

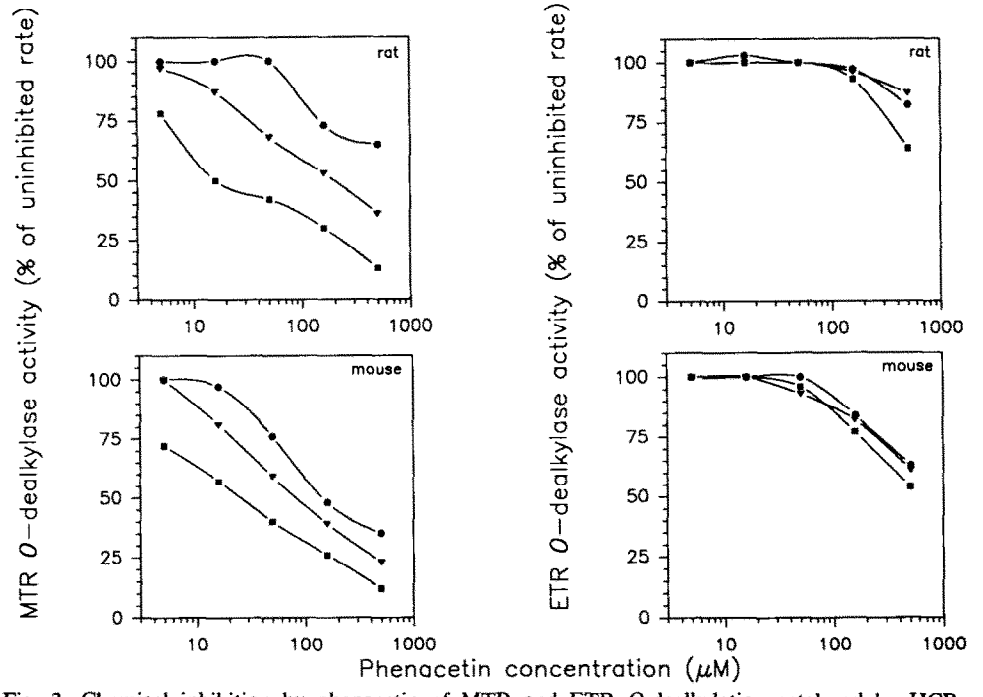

Fig. 3. Chemical inhibition by phenacetin of MTR and ETR *O*-dealkylation catalyzed by HCB-pretreated rat and TCDD-pretreated mouse S-9 preparations. Substrate concentrations were (●) 5 μM, (▼) 1.75 μM, and (■) 0.5 μM. Uninhibited MTR and ETR *O*-dealkylation activities were 420 and 1700 pmol/min/mg S-9 protein, respectively, in the rat, and 1800 and 2200 pmol/min/mg S-9 protein, respectively, in the mouse.

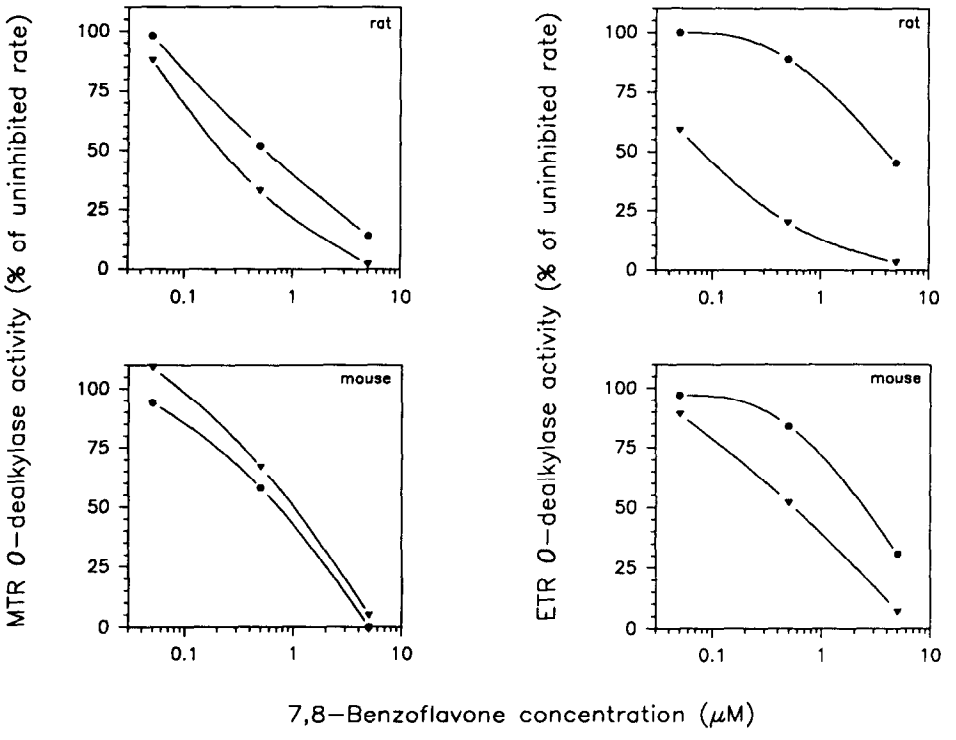

Fig. 5. Chemical inhibition by 7,8-benzoflavone of MTR and ETR *O*-dealkylation catalyzed by HCB-pretreated and control rat and TCDD-pretreated and control mouse S-9 preparations. The substrate concentration was 5 μM. Uninhibited MTR and ETR *O*-dealkylation activity values (in pmol/min/mg S-9 protein) were 420 and 1700, respectively, for HCB-pretreated rats (▼); 21 and 36, respectively, for control rats (●); 1800 and 2200, respectively, for TCDD-pretreated mice (▼); and 13 and 21, respectively, for control mice (●).

In contrast, the k_i was ≥ 333 μM at an ETR concentration of 0.5 μM (Fig. 3). Similar k_i values were obtained for the inhibition by phenacetin of the corresponding reactions in the presence of mouse S-9.

The ability of the CYP1A inhibitor, 7,8-benzoflavone (Fig. 1), to inhibit ETR and MTR *O*-dealkylation activities in S-9 samples from control and TCDD-induced mice and HCB-treated rats is presented in Fig. 5 and Table 3. MTR and ETR *O*-dealkylation activities in hepatic S-9s from induced mice and rats were equally sensitive to the inhibitory activity of 7,8-benzoflavone. However, ETR metabolism in HCB-induced rats was highly sensitive to inhibition by 7,8-benzoflavone, whereas this activity in control rats was two orders of magnitude less sensitive. This differential inhibition by 7,8-benzoflavone was not nearly as striking in mice. Thus, MTR *O*-dealkylation activity was inhibited by 7,8-benzoflavone to an equivalent extent in S-9 from control and induced animals of either species.

The ability of monoclonal antibody C-8 (directed against CYP1A1, but not cross-reactive with CYP1A2) to inhibit hepatic MTR and ETR *O*-dealkylation activities is shown in Fig. 6. Profound (~75%) inhibition of ETR metabolism was observed at an antibody/protein ratio of 1 : 1. In contrast, < 2% inhibition of MTR metabolism by this monospecific antibody was observed. Monoclonal antibody 7-1-1, which is cross-reactive with both CYP1A1 and CYP1A2, inhibited the two *O*-dealkylation activities to the same extent, in both species of rodents.

Induction and immunoinhibition studies were also performed in order to determine the isozyme specificity of the *O*-dealkylation of BZR in the mouse and rat. While MTR and ETR *O*-dealkylation activities were only weakly (< 4-fold) induced by PB, PCN or DEX in both rats and mice, BZR *O*-dealkylation and PTR *O*-dealkylation activities were highly (> 35-fold) induced in the two species by the prototype CYP2B inducer, PB. BZR and PTR *O*-dealkylation activities were highly (> 40-fold) induced by DEX in mice but the same treatment regimen in rats yielded a weaker response (~13-fold). PCN administration resulted in relatively minimal (3- to 5-fold) induction of these activities. In the immunoinhibition studies (Fig. 7), it was demonstrated that BZR and PTR *O*-dealkylation activities in microsomes from PB- or DEX-pretreated rats and mice were inhibited by antibody reactive against CYP2B but not by antibody reactive to CYP1A.

The induction of various alkoxyresorufin *O*-dealkylation activities in B6C3F1 mice exposed for 2 weeks to dietary Aroclor 1254 is shown in Table 4. Although Aroclor concentration-dependent increases in *O*-dealkylation activities for each of the substrates were observed, the dose–response curves

Table 3. Effects of 7,8-benzoflavone on the metabolism of MTR and ETR in S-9 from control or xenobiotic-pretreated rodents

Rodent	Pretreatment†	k_i (μM) for inhibition by 7,8-benzoflavone*: MTR *O*-dealkylation	ETR *O*-dealkylation
Mouse	Control	0.4	0.5
	TCDD	0.5	0.1
Rat	Control	0.6	5.0
	HCB	0.4	0.05

* k_i values for 7,8-benzoflavone were determined in S-9 in the presence of 2 μM MTR or ETR. Preliminary results indicated non-competitive inhibition by 7,8-benzoflavone (data not shown).

† Abbreviations and dosage regimens used: TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) was administered as a single i.p. injection of 16 μg/kg body weight; HCB (3,4,5,3′,4′,5′-hexachlorobiphenyl) was administered at 8 ppm in the diet for 5 days.

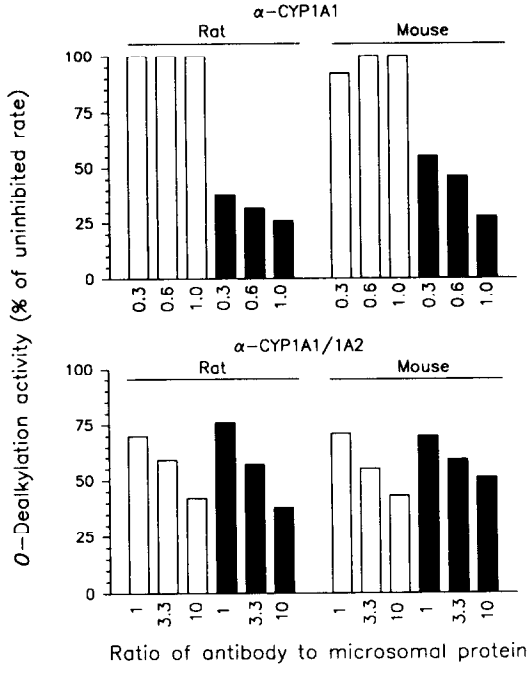

Fig. 6. Immunoinhibition of MTR (open bars) and ETR (solid bars) *O*-dealkylation catalyzed by HCB-pretreated rat and TCDD-pretreated mouse microsomal preparations. Monoclonal antibodies inhibitory for CYP1A1 (α-CYP1A1, MAb C8 [19, 20]) or inhibitory for CYP1A1/1A2 (α-CYP1A1/1A2, MAb 1-7-1 [21]) were incubated at the indicated antibody to microsomal protein ratio for 20 min. Uninhibited MTR and ETR *O*-dealkylation activities were 1800 and 7300 pmol/min/mg microsomal protein, respectively, in the rat, and 7400 and 9600 pmol/min/mg microsomal protein, respectively, in the mouse.

for the three substrates were not superimposable. Thus, statistically significant increases in MTR *O*-dealkylation activity occurred at Aroclor concentrations as low as 1.0 ppm, whereas concentrations of 33 ppm were required for significant increases in ETR or BZR *O*-dealkylation activity.

DISCUSSION

The alkoxyresorufins have proven to be rather sensitive and relatively isozyme-specific substrates for a number of vertebrate and invertebrate CYP proteins. As early as the mid-1970s, ETR metabolism was shown to be highly induced by 3-methylcholanthrene in rats and to be preferentially mediated by CYP1A1 [26]. More recently, PTR has been shown to be a specific substrate for CYP2B in mice, rats and rabbits [14, 15, 27–29]. In contrast, the isozyme specificity of MTR and BZR has been less adequately defined. MTR, which has been useful for studying invertebrate CYP [30], has only more recently been employed as a model substrate in mammals. Namkung *et al.* [14] have presented data which indicate that MTR is a highly specific substrate for rat CYP1A2, and the use of this substrate is gaining acceptance as a means of probing for this isozyme [31]. This finding is of particular interest due to the fact that CYP1A2 has been found to be involved in the activation of a number of procarcinogenic aromatic amines in both the human as well as laboratory animal species [8–10], and the existing assays for this isozyme have been difficult to perform and/or rather non-specific.

In contrast to the rather limited use of MTR to date, BZR has been employed extensively as a CYP substrate. However, there has been considerable disagreement regarding the isozyme specificity of the *O*-dealkylation of this substrate. This activity, while highly induced by phenobarbital, has also been reported to be induced by isosafrole, a known inducer of CYP1A2 [15]. Based upon this, it has been hypothesized that this resorufin ether may be preferentially metabolized by CYP1A2 [15]. Further, Namkung *et al.* [14] have shown that BZR metabolism is inducible by the CYP3A1-type inducers PCN and DEX. In addition, these authors observed metabolism of this resorufin ether by purified CYP2B1/2B2, 1A1 and, to a lesser extent, by 1A2

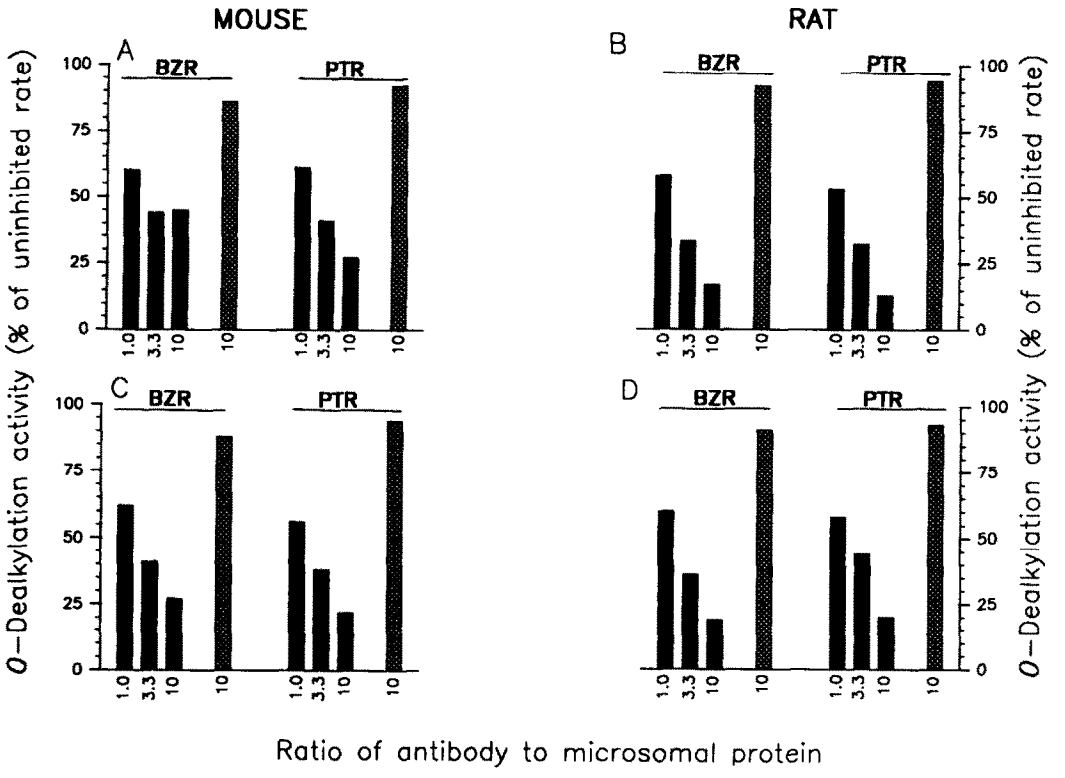

Fig. 7. Immunoinhibition of BZR and PTR *O*-dealkylation catalyzed by rat and mouse microsomal preparations from phenobarbital- (panels A and B) or dexamethasone- (panels C and D) pretreated animals. Monoclonal antibodies inhibitory for CYP2B1/2B2 (MAb 2-66-3 [22], solid bars) or inhibitory for CYP1A1/1A2 (MAb 1-7-1 [21], hatched bars) were incubated at the indicated antibody to microsomal protein ratio for 20 min. Uninhibited BZR and PTR *O*-dealkylation activities were 4400 and 1900 pmol/min/mg microsomal protein, respectively, for PB-pretreated rats, 1400 and 690 pmol/min/mg, respectively, for DEX-pretreated rats, 1300 and 490 pmol/min/mg microsomal protein, respectively, for PB-pretreated mice, and 1400 and 490 pmol/min/mg, respectively, for DEX-pretreated mice.

Table 4. Induction of alkoxyresorufin *O*-dealkylation activities in B6C3F1 mice exposed to dietary Aroclor 1254

Dietary Aroclor concentration* (ppm)	Alkoxyresorufin *O*-dealkylation activity† (pmol resorufin formed/min/mg S-9 protein)		
	MTR	ETR	BZR
0	9.2 ± 0.7	21 ± 2	7.5 ± 1.8
1.0	14 ± 2‡	23 ± 4	7.8 ± 1.7
3.3	16 ± 1‡	19 ± 1	7.7 ± 1.5
10	59 ± 12‡	19 ± 5	8.9 ± 1.9
33	160 ± 33‡	110 ± 20‡	36 ± 12‡
100	820 ± 160‡	700 ± 130‡	260 ± 41‡
333	2900 ± 410‡	2700 ± 88‡	590 ± 86‡
500	2800 ± 310‡	3500 ± 500‡	590 ± 120‡

* Mice were fed the indicated dietary concentrations of Aroclor 1254 for 14 days.

† Values are means ± SD for four mice/treatment. All values have been rounded to two significant figures.

‡ Significantly different from control group ($P < 0.05$).

[14]. These facts and others have led the authors to conclude that "many if not most of P450 isozymes will catalyze debenzylation of benzyloxyphenoxazone" [14]. In fact, the finding that purified CYP2B can metabolize BZR at rates only somewhat higher than those for other purified CYP proteins may reflect the fact that purified cytochrome P450 reductase can chemically reduce BZR and PTR, effectively blocking their *O*-dealkylation [32]. This phenomenon results in a striking underestimate of the rate of *O*-dealkylation by these particular purified CYPs. The work of Meehan *et al.* [28] would appear to show that the dealkylation of both PTR and BZR is preferentially mediated by CYP2B in both mice and rats. We undertook the present series of experiments in order to clarify these somewhat contradictory results of previous investigators with respect to the isozyme specificity of the MTR and BZR *O*-dealkylation reactions.

We examined the role of CYP1A1 and 1A2 in mediating MTR and ETR *O*-dealkylation activities by three different methods: (a) induction studies in hepatic and extrahepatic tissues, (b) chemical inhibition studies, and (c) immunochemical inhibition studies.

It has been shown previously that CYP1A1 is expressed at very low levels in both liver and extrahepatic tissues of untreated mice, but is highly inducible in these tissues. In contrast, CYP1A2 is constitutively expressed and is inducible in hepatic tissues, but only minimally expressed or inducible in extrahepatic tissue [1, 12]. Similarly, in western blot analyses employing monoclonal antisera cross-reactive with both CYP1A1 and CYP1A2 performed in the present study, both CYP1A proteins were highly induced in liver microsomes, whereas only a single immunoreactive protein (CYP1A1) was induced in the kidney. In our catalytic studies, the metabolism of ETR was highly induced in the livers of both rats and mice. Maximal levels in excess of 1500 pmol/min/mg S-9 protein were achieved. This activity was also highly induced (> 250-fold, to levels of 260 pmol/min/mg) by TCDD in the kidneys of rats but substantially less induction (~30-fold, to levels of 31 pmol/min/mg) was detected in TCDD-treated mouse kidney. The metabolism of MTR was maximally induced 26-fold in the rat liver (to levels of 550 pmol/min/mg) and 180-fold in the mouse liver (to levels of 2500 pmol/min/mg). The high degree of induction of *O*-dealkylation of MTR in mice by polyhalogenated aromatic hydrocarbons was observed in two different strains of mice (NIH/Swiss and B6C3F1), implying that at least in the case of these two strains of mouse, MTR is an effective substrate for probing CYP1A2. There are, however, known genetic polymorphisms in the CYP1A2 structural gene [33] which potentially may alter the substrate specificity of the isozyme. In contrast to the high levels of MTR *O*-dealkylation activity present in induced liver, maximally induced levels of this activity in the kidney were 16 and 10 pmol/min/mg (representing 30- and 10-fold induction) in the rat and mouse, respectively. These results provide indirect evidence that the metabolism of MTR is preferentially mediated by CYP1A2, while ETR metabolism is primarily mediated by CYP1A1.

When compared with previously reported induction of CYP1A2 in the rat, monitored either by phenacetin *O*-deethylation activity or metabolism of certain tryptophan pyrolysis products, our observation of 30-fold induction of MTR *O*-dealkylation activity would appear to be in rough agreement [10–12]. These results are also in agreement with the ~20-fold induction of CYP1A2, determined with immunologic procedures, which is observed following pretreatment of rats with a variety of agents [34] and which we observed in the present studies. Our immunoblotting data in mice, which implies 80- to 100-fold induction of protein, is in agreement with the ~150-fold increase in catalytic activity for MTR which was observed.

Phenacetin, a substrate for CYP1A2 that displays a relatively low K_m in both rats and humans [11, 12], proved to be an effective competitive inhibitor of MTR *O*-dealkylation activity in both mice and rats, while being markedly less effective as an inhibitor of ETR *O*-dealkylation activity. These results imply that different P450 isozymes mediate the two activities. 7,8-Benzoflavone strongly inhibited both of these activities. Interestingly, for both mice and rat S-9 samples, 7,8-benzoflavone inhibited MTR *O*-dealkylation in control and induced samples to an equal extent. This result could be taken to imply that both the constitutive and induced MTR *O*-dealkylation activities are mediated by the same or similar cytochromes. In contrast, there were more distinct differences in the inhibition of ETR *O*-dealkylation activity by 7,8-benzoflavone. Thus the k_i for ETR *O*-dealkylation activity measured in S-9 samples from control rats was 5.0 μM, while the k_i in highly induced S-9 from HCB-pretreated rats was 0.05 μM. This trend towards differential inhibition of ETR *O*-dealkylation activity by 7,8-benzoflavone in control and xenobiotic-pretreated animals was less apparent in mice (k_i: control, 0.5 μM; TCDD-pretreated, ~0.15 μM). These results strongly imply that ETR *O*-dealkylation activity is mediated by different CYP proteins in control versus xenobiotic-pretreated rats, as previously demonstrated by Kelley and co-workers [35], employing specific monoclonal antibodies.

Immunoinhibition studies employing monoclonal antibodies [19–22] were conducted to examine further the isozyme specificity of these *O*-dealkylation reactions. Both MTR and ETR *O*-dealkylation activities were inhibited by MAb 1-7-1, which has been shown to inhibit both CYP1A1 and CYP1A2 [21]. On the other hand, MAb C-8, which reacts with and inhibits the catalytic activity of rat CYP1A1 but not CYP1A2 [19, 20], caused inhibition of ETR *O*-dealkylation but did not inhibit the metabolism of MTR in samples from mice or rats.

The P450 isozyme specificity of the *O*-dealkylation of BZR was examined in order to clarify the conflicting literature regarding this substrate. BZR and PTR *O*-dealkylation activities were highly induced by PB in the rat and mouse but more limited induction resulted from the prototype CYP3A-type (PCN) or CYP1A-type (TCDD, 3,4,5,3′,4′,5′-HCB) inducers. The minimal increases in BZR metabolism which result from HCB pretreatment in rats are not accompanied by detectable increases in CYP2B1

protein or RNA [34], and most likely represent the rather weak catalytic activity (for BZR metabolism) of CYP1A, which is highly induced by this treatment. This is supported by the fact that the BZR *O*-dealkylation activity in HCB-pretreated rat S-9 was inhibited by monoclonal antibodies directed against CYP1A and was sensitive to inhibition by 7,8-benzoflavone (data not shown). Namkung *et al.* [14] and Burke *et al.* [15] have suggested that BZR is metabolized by a variety of CYP proteins, while Chen and Eaton [16] have hypothesized that this resorufin ether may represent a model substrate for CYP3A. However, we have found that strong inducers of CYP3A (e.g. PCN) are rather weak inducers of BZR *O*-dealkylation activity (≈15% of maximal PB-type induction). In contrast, DEX appears to induce this activity more strongly, particularly in the mouse. However, we have shown in immunochemical detection studies that DEX induced CYP2B protein in the rat and even more strongly in the mouse (Nims RW and Lubet RA, unpublished data), a result that was initially observed by Meehan and co-workers [28] and which has been confirmed more recently by Corcos [36]. Our inhibition studies in S-9 samples from both PB- and DEX-pretreated animals support the primary role of CYP2B in *O*-dealkylation of BZR. One should be aware that the dose of DEX employed in the present studies was non-physiologic and was somewhat toxic, as evidenced by the 18% decrease in body weight gained over the 5-day period in the DEX-treated rats. In contrast, this dose of DEX did not affect significantly body weights in mice, compared with vehicle controls. Nevertheless, this dose of DEX did cause maximal induction of CYP3A when compared to the response caused by other CYP3A inducers such as PCN and clotrimazole.

Finally, we employed the various alkoxyresorufin *O*-dealkylation assays to determine indirectly the induction of CYP isozymes in mice following dietary exposure to Aroclor 1254. A concentration-dependent increase in the rate of metabolism of MTR, ETR and BZR was observed over the range of concentrations examined (1–500 ppm Aroclor 1254). However, MTR *O*-dealkylation activity was increased at lower Aroclor concentrations than those required for induction of ETR or BZR *O*-dealkylation activity. This result was to be expected if MTR *O*-dealkylation is mediated primarily by CYP1A2, as Ikeda *et al.* [37] have shown that the 3-methylcholanthrene dose–response curve for induction of mouse $P_2$450 (CYP1A2) is shifted to the left in comparison to the curve for $P_1$450 (CYP1A1).

In summary, the present experiments confirm the use of these various alkoxyresorufin assays as simple, sensitive and relatively selective assays for monitoring specific CYP activities in these two rodent species. CYP1A2 appears to preferentially metabolize MTR *O*-dealkylation, CYP1A1 preferentially metabolizes ETR *O*-dealkylation, and CYP2B1/2 preferentially metabolizes the *O*-dealkylation of both BZR and PTR. Preliminary studies in non-human primates [38] yield more variable results. Thus, BZR *O*-dealkylation activity is only weakly induced by PB treatment in the patas or cynomolgus monkey, while PTR *O*-dealkylation activity is highly induced in some PB-treated individuals but not in others, suggesting that a polymorphism exists with respect to this trait. Indeed, hydroxylation of testosterone at the 16β-position may prove the most generally applicable substrate for CYP2B in non-human primates [38]. In addition, preliminary results in primates imply that both CYP1A1 and CYP1A2 may metabolize both MTR and ETR (Lubet RA, data not shown).

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