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EVIDENCE FOR MECHANISM-BASED INACTIVATION OF RAT AND CHICK EMBRYO HEPATIC CYTOCHROME P4501A AND P4503A BY DIHYDROPYRIDINES, SYDNONES, AND DIHYDROQUINOLINES

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Abstract—Rat hepatic P4501A1 and 3A1/2 have been shown previously to be targets for mechanism-based inactivation by the 4-alkyl analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), namely, 4-ethyl DDC and 4-isopropyl DDC. In this study we have shown that rat hepatic P4501A and P4503A are targets for mechanism-based inactivation by the sydnones, 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone (TTMS) and 3-(2-phenylethyl)-4-methylsydnone (PEMS). The dihydroquinoline, 2,4-diethyl-2-methyl-1,2-dihydroquinoline (DMDQ), caused mechanism-based inactivation of rat hepatic P4501A but not of P4503A. The P4501A isozyme(s) of chick embryo liver was found to share the ability of rat liver P4501A to serve as a target for mechanism-based inactivation by the dihydropyridines, 4-ethyl DDC and 4-isopropyl DDC, the sydnones, TTMS and PEMS, and the dihydroquinoline, DMDQ. A P4503A-like isozyme of chick embryo liver shared the ability of the rat liver P4503A isozyme(s) to serve as a target for mechanism-based inactivation by the dihydropyridines, 4-ethyl DDC and 4-isopropyl DDC, and the sydnone, TTMS, but not of the sydnone PEMS. The dihydropyridine, DDC, was found to serve as a mechanism-based inactivator of the chick embryo P4501A isozyme(s), but not of the P4503A isozyme(s), in contrast to its previously reported inactivity with both the rat hepatic P4501A1 and 3A1/2 isozymes.

Key words: porphyria, drug induced; mechanism-based inactivation; cytochrome P450 isozymes; dihydropyridines; sydnones; chick embryo

The dihydropyridine, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC)† and various 4-alkyl analogues (Fig. 1a) [1–7], the sydnones, 3-[2-(2,4,6-trimethylphenyl)thioethyl] -4-methylsydnone (TTMS) (Fig. 1b, compound ii) and 3-(2-phenylethyl)-4-methylsydnone (PEMS) (Fig. 1b, compound i) [8], and the dihydroquinolines, 2,4-diethyl-2-methyl-1,2-dihydroquinoline (DMDQ) and 2,2,4-trimethyl-1,2-dihydroquinoline (TMDQ) (Fig. 1c) [9], are porphyrinogenic chemicals that cause mechanism-based inactivation of cytochrome P450 (P450) (EC 1.14.14.1). The key structural requirement in this group of compounds for ferrochelatase inhibition, resulting in 5-aminolevulinic acid synthase (ALAS) induction and porphyrin accumulation, is a heterocyclic ring, which, when rendered unstable following oxidation of P450, releases a substituent that causes prosthetic heme *N*-alkylation [2]. The result, in most cases, is the release of iron from the *N*-alkylheme and the production of a mixture of the four regioisomers (i.e. the N_A, N_B, N_C, and N_D regioisomers) of the corresponding *N*-alkylprotoporphyrin IX (*N*-alkylPP). The *N*-alkylPPs, particularly the N_A and N_B regioisomers [10, 11], are potent inhibitors of ferrochelatase (EC 4.99.1.1), the terminal enzyme of the heme biosynthetic pathway. This inhibition results in disruption of the heme biosynthetic pathway and the accumulation of protoporphyrin IX.

The P450 enzymes targeted by dihydropyridines for mechanism-based inactivation in rat liver have been shown to be P4501A1, 3A1/2, 2C6, and 2C11 [5, 12–14]. The mechanism-based inactivation includes the destruction of heme and *N*-alkylPP formation from the heme moiety or moieties of P450 isozymes. It is logical to deduce that the ferrochelatase inhibitory *N*-alkylPPs are derived from one or several rat hepatic P450 isozymes. There is little information available for the P450 targets of sydnones and dihydroquinolines. It is therefore of interest to determine how the P450 targets of the sydnones and dihydroquinolines relate to those for the dihydropyridines, and this constituted the first objective of this study.

Data concerning the porphyrinogenicity of dihydropyridines, sydnones, and dihydroquinolines

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† Abbreviations: DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; TTMS, 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone; PEMS, 3-(2-phenylethyl)-4-methylsydnone; DMDQ, 2,4-diethyl-2-methyl-1,2-dihydroquinoline; TMDQ, 2,2,4-trimethyl-1,2-dihydroquinoline; P450, cytochrome P450; *N*-alkylPP, *N*-alkylprotoporphyrin IX; PB, phenobarbital; βNF, β-naphthoflavone; DEX, dexamethasone; 7ERFOD, 7-ethoxyresorufin *O*-deethylase; ERND, erythromycin *N*-demethylase; and HCHO, formaldehyde.

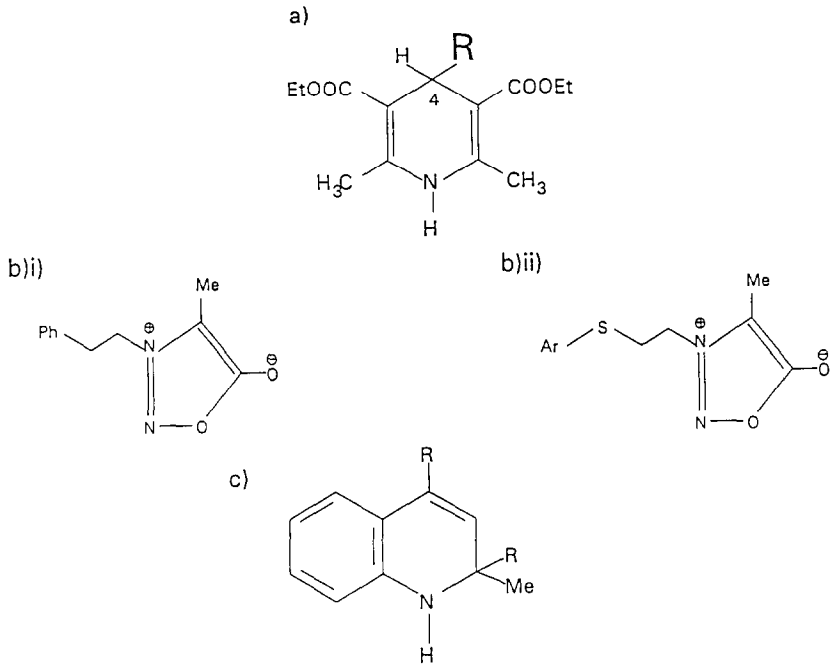

Fig. 1. Structure of: (a) the dihydropyridine analogues. (i) R = —CH_3, DDC; (ii) R = —CH_2CH_3, 4-ethyl DDC; (iii) R = —$CH(CH_3)_2$, 4-isopropyl DDC; (b) the sydnones. (i) 3-(2-phenylethyl)-4-methylsydnone (PEMS); (ii) 3-[2-(2,4,6-trimethylphenyl) thioethyl]-4-methylsydnone (TTMS); (c) the dihydroquinolines. (i) R = CH_2CH_3, 2,4-diethyl-2-methyl-1,2-dihydroquinoline (DMDQ); (ii) R = CH_3, 2,2,4-trimethyl-1,2-dihydroquinoline (TMDQ).

have been obtained from studies using chick embryo hepatocyte cultures [1–4, 8, 9]. In one study, one hundred phenobarbital (PB)-treated chick embryos, injected *in vivo* with 4-ethyl DDC, were required for the isolation and identification of *N*-ethylprotoporphyrin [15]. This was due to the requirement for sufficient liver tissue in order to isolate, identify, and separate the regioisomers of *N*-alkylPPs. Therefore, in the remainder of the studies concerning *N*-alkylPPs, porphyrinogenic compounds were administered to a small number of PB-treated rats, and rodent liver was utilized for the *N*-alkylPP isolation. Since the P450 isozymes differ between chick embryo and rat liver [16–18], it is of interest to determine which of the rat hepatic P450 isozyme targets of porphyrinogenic drugs are present in chick embryo liver and constitute targets for these drugs.

It may be deduced from evidence summarized in a review by Sinclair and Sinclair [18] that dexamethasone induces a form of cytochrome P450 in young chickens that might be cytochrome P4503A. This isozyme(s) was found to cross-react with a monoclonal antibody to rat cytochrome P4503A [19], and its induction was parallelled by increased erythromycin *N*-demethylase (ERND) activity, which serves as a marker for rat cytochrome P4503A [20]. This isozyme(s) is induced by PB in 14- and 19-day-old chick embryos and after hatching [19]. Moreover, PB has also been shown in chick embryo liver to induce testosterone-6 β-hydroxylase [21], another enzyme activity associated with the cytochrome P4503 family [22]. These reviewers [18] also reported that a cytochrome P450 isozyme(s) similar to those of the 1A subfamily has been induced in chick embryo liver by planar aromatic hydrocarbons. Therefore, in the current study, we refer to the isozymes in the chick embryo as P4501A and 3A, with the understanding that further work is necessary to completely delineate the nature of these isozymes.

The second objective of our study was to determine whether cytochrome P4501A and 3A of chick embryo liver were targets for mechanism-based inactivation by dihydropyridines, as had been shown to be the case with rat liver P4501A1 and 3A1/2. Moreover, it was of interest to determine if these chick embryo liver isozymes were also targets for sydnones and dihydroquinolines.

MATERIALS AND METHODS

Source of chemicals. TTMS, DMDQ, and TMDQ were obtained from Colour Your Enzyme, (Bath, Ontario). PEMS was a gift of Dr. P. R. Ortiz de Montellano (Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA). DDC analogues were synthesized in this laboratory by the method of Loev and Snader [23] as described previously [3, 24, 25]. Chemicals were purchased from the following sources: sodium phenobarbital (PB), (British Drug Houses Inc., Toronto, Ontario); β-naphthoflavone (βNF) and dexamethasone (DEX), (Aldrich Chemical Co., Milwaukee, WI); resorufin and 7-ethoxyresorufin, (Molecular Probes Inc., Eugene, OR);

and NADPH and erythromycin, (Sigma Chemical Co., St. Louis, MO).

Source of animals. Fertilized eggs of the White Leghorn strain were obtained from Archer's Poultry Farm (Brighton, Ontario). Eggs were stored at 4° for no more than 9 days prior to incubation in a Humidaire Incubator (New Madison, OH) at 37° with a humidity of 86%. Embryo age was considered to be the number of days from the onset of incubation.

Male Sprague–Dawley rats (250–300 g) were obtained from Charles River Canada Inc. (St. Constant, Quebec). The rats were fed Purina Laboratory Rat Chow (No. 5001) and water *ad lib.* and were housed under controlled conditions (22°, 14 hr light/10 hr dark cycle).

Induction of chick embryo hepatic microsomal cytochrome P450 and preparation of microsomes. Chick embryos received no treatment or were injected through a small hole made in the egg shell above the air sac using a 1-in. 20-gauge needle and a 1-mL Tuberculin syringe. PB was administered in 0.1 mL of deionized water (6.0 mg/egg/day for 2 days), βNF in 0.1 mL of DMSO (2.0 mg/egg for 1 day), and DEX in 0.1 mL of DMSO (5.0 mg/egg for 1 day) such that all the chick embryos had been incubated for 19 days at the end of the treatment period. Following drug administration, the hole in the egg shells was sealed with sellotape and the eggs were returned to the incubator.

The 19-day-old chick embryos were killed by decapitation, and their livers were removed and rinsed in ice-cold 10 mM potassium phosphate buffer (K_2HPO_4) (pH 7.4) containing 1.15% KCl. The livers were homogenized in the buffered KCl, and the microsomes were isolated by differential centrifugation, as described previously [1]. The final microsomal pellet was frozen at −70° for up to 2 weeks [26].

Induction of rat hepatic microsomal cytochrome P450 and preparation of microsomes. Rats were injected intraperitoneally with βNF in corn oil (40 mg/kg/day for 3 days), PB in deionized water (80 mg/kg/day for 4 days), or DEX in corn oil (100 mg/kg/day for 4 days), and were killed by decapitation 24 hr after the final treatment. Livers were perfused *in situ* with ice-cold 1.15% KCl (50 mL/rat), excised, minced with scissors, and homogenized in 4 vol. of ice-cold 10 mM K_2HPO_4 (pH 7.4) containing 1.15% KCl. Microsomes were isolated by differential centrifugation, as described previously [13]. The final microsomal pellet was frozen at −70° for up to 2 weeks.

Mechanism-based inactivation of cytochrome P450. The general procedure has been reported previously [13]. Microsomal pellets were resuspended in 0.1 M K_2HPO_4 (pH 7.4) containing 1.5 mM EDTA, to yield approximately 2–4 mg/mL microsomal protein. The microsomal suspension (2.0 mL) was added to 25-mL Erlenmeyer flasks containing 2.0 mL of a 2.0 mM NADPH solution in the above incubation buffer. In the chick embryo preparations, DDC, 4-ethyl DDC, 4-isopropyl DDC, PEMS, TTMS, DMDQ, or TMDQ was added to this suspension, resulting in a final concentration of 45 μM. In the rat preparations, 4-ethyl DDC, PEMS, TTMS, DMDQ, or TMDQ was added to the suspension, resulting in a final concentration of 45 μM. For studies in the rat in which mechanism-based inactivation of P450 was to be followed by an isozyme-selective catalytic assay, higher concentrations of sydnones (250 μM) and dihydroquinolines (100 μM) were employed. In all experiments, the following controls were run concurrently: (i) omission of NADPH; (ii) omission of the inactivator; and (iii) omission of NADPH and the inactivator, in chick embryo liver microsomes only. Samples were incubated for 30 min in a 37° shaking water bath, and the reaction was terminated by cooling the samples on ice. P450 levels were determined spectrophotometrically by the method of Omura and Sato [27], as described previously [1], with the exception of DEX-treated microsomes, where P450 levels were measured by the method of Estabrook *et al.* [28]. Protein was assayed by the method of Lowry *et al.* [29].

Measurement of cytochrome P450 isozyme-selective catalytic activities. Following the treatment of microsomal P450 by the dihydropyridines, sydnones, or dihydroquinolines, as described above, the microsomes were reharvested by centrifugation at 106,000 *g* for 1 hr at 4°. Microsomal pellets were assayed for 7-ethoxyresorufin *O*-deethylase (7ERFOD) activity according to the method of Burke *et al.* [30], or ERND activity according to the method of Wrighton *et al.* [20]. These assays have been described previously by Riddick *et al.* [13].

Statistical analysis. A repeated-measures design one-way analysis of variance was used to determine whether two means differed significantly from each other ($P \leq 0.05$). If a significant F ratio at the 0.05 level was obtained, a Newman–Keuls test was used to indicate the means that differed significantly from each other.

For the results to be considered significant, the activity of the drug in the presence of NADPH had to be significantly different from that measured in control (−drug, +NADPH) and that measured in the presence of drug but absence of NADPH.

RESULTS AND DISCUSSION

Choice of concentrations of chemicals employed for mechanism-based inactivation studies. In previous studies [13], concentrations of 450 μM were used for studies of P450 loss following the incubation of dihydropyridines with rat hepatic microsomes. While this concentration was found to be appropriate for these studies, the concentration was found to be too high for studies of catalytic function following mechanism-based inactivation of the microsomes. Since the dihydropyridines are highly lipophilic, a sufficient residual amount of these compounds may have remained in the microsomes resulting in competitive inhibition of 7-ethoxyresorufin employed in the 7ERFOD assay or erythromycin employed in the ERND assay. Reduction of the dihydropyridine concentration from 450 to 45 μM allowed mechanism-based inactivation of P450 to occur without interference with the subsequent measurement of 7ERFOD or ERND activity. From our preliminary studies on mechanism-based inactivation of rat hep-

atic microsomes with sydnones and dihydroquinolines, we selected concentrations of 250 μM for the sydnones and 100 μM for the dihydroquinolines for the determination of 7ERFOD and ERND activity; 4-ethyl DDC (45 μM) was chosen as a positive control on the basis of previous experience [13]. Due to the inhibition observed with the sydnones and dihydroquinolines on 7ERFOD and ERND activity of rat hepatic microsomes, lower doses, namely 45 μM, were selected for all subsequent studies.

Effect of dihydropyridines, sydnones, and dihydroquinolines (45 μM) on cytochrome P450 content of rat hepatic microsomes. In microsomes from DEX-treated rats, P450 levels determined by the method of Omura and Sato [27] were found to be significantly higher following incubation in the presence of drug (−NADPH), as compared with incubation in the presence of control (−drug, +NADPH) (results not shown). Previous workers [13] found similar results with DEX-treated rats, and suggested that despite the inclusion of 1.5 mM EDTA in the microsomes, NADPH-dependent lipid peroxidation was occurring. Using the method of Buege and Aust [31] to assess lipid peroxidation in microsomes by measuring malondialdehyde production, these workers [13] were able to demonstrate that DEX-treated rat microsomes, but not untreated, PB- or βNF-treated microsomes, produced significant amounts of malondialdehyde during a 30-min incubation in the presence of 1.0 mM NADPH. There is sufficient CO generated by lipid peroxidative processes to cause greater artifactual spectral losses of P450 than those actually occurring [32], when the Omura and Sato method [27] for P450 measurements is employed. Therefore in the current study, P450 levels were measured by the Omura and Sato method [27] with the exception of DEX-treated microsomes, where P450 levels were measured by the method of Estabrook *et al.* [28] to eliminate the spectral problem caused by residual CO.

The P450 loss in hepatic microsomes from untreated, βNF-, PB-, and DEX-treated rats is shown in Table 1. 4-Ethyl DDC and TTMS caused an NADPH-dependent loss of P450 in microsomes from untreated, βNF-, PB-, and DEX-treated microsomes. PEMS caused an NADPH-dependent loss of P450 in βNF-, PB-, and DEX-treated microsomes, but not in untreated microsomes. Mechanism-based inactivation of P450 has been reported previously in PB-treated rat hepatic microsomes with both TTMS and PEMS [33, 34]. DMDQ caused an NADPH-dependent loss of P450 in DEX-treated microsomes only, while TMDQ was inactive in all microsomal preparations.

Mechanism-based inactivation of P4501A in rat hepatic microsomes assessed using 7ERFOD activity as a catalytic marker. Following the incubation of microsomes with NADPH and a dihydropyridine, sydnone, or dihydroquinoline, the microsomes were harvested by ultracentrifugation (100,000 *g*) and assayed for functional activity. Mechanism-based inactivation of P4501A was examined by measuring 7ERFOD activity in βNF-induced rat hepatic microsomes (Fig. 2). All the drugs tested, except TMDQ, caused a depression of 7ERFOD activity in the presence of NADPH that was significantly different from that measured in control (−drug, +NADPH) and that measured in the presence of drug but absence of NADPH. This NADPH-dependent inhibition shows that P4501A is a target for mechanism-based inactivation in the rat. Incubation of microsomes from βNF-treated rats with all of the drugs tested in the absence of NADPH resulted in significant inhibition of 7ERFOD activity as compared with the control (−drug, +NADPH) (Fig. 2). This reduction in

Table 1. Effects of 4-ethyl DDC, the sydnones, and the dihydroquinolines (45 μM) on cytochrome P450 content of hepatic microsomes from untreated, βNF-, PB-, and DEX-treated rats

Drug	Cytochrome P450 content (nmol/mg protein)			
	Untreated*	βNF-treated*	PB-treated*	DEX-treated†
Control	0.90 ± 0.12	1.28 ± 0.24	1.55 ± 0.50	0.98 ± 0.08
Control + NADPH	0.81 ± 0.21	1.10 ± 0.21	1.31 ± 0.55	0.91 ± 0.02
4-Ethyl DDC	0.97 ± 0.19	1.29 ± 0.21	1.56 ± 0.52	1.00 ± 0.03
4-Ethyl DDC + NADPH	0.48 ± 0.07‡	0.62 ± 0.11‡	0.82 ± 0.37‡	0.53 ± 0.08‡
TTMS	1.02 ± 0.18	1.24 ± 0.20	1.55 ± 0.54	1.02 ± 0.06
TTMS + NADPH	0.67 ± 0.19‡	0.82 ± 0.16‡	0.74 ± 0.30‡	0.27 ± 0.04‡
PEMS	0.96 ± 0.26	1.29 ± 0.17	1.60 ± 0.57	0.97 ± 0.08
PEMS + NADPH	0.73 ± 0.19	0.87 ± 0.15‡	1.01 ± 0.42‡	0.76 ± 0.04‡
DMDQ	1.05 ± 0.25	1.41 ± 0.26	1.60 ± 0.51	1.03 ± 0.10
DMDQ + NADPH	0.82 ± 0.27	1.09 ± 0.26	1.18 ± 0.43	0.77 ± 0.10‡
TMDQ	1.00 ± 0.26	1.39 ± 0.14	1.61 ± 0.48	1.02 ± 0.03
TMDQ + NADPH	0.88 ± 0.22	1.06 ± 0.19	1.48 ± 0.52	0.97 ± 0.04

All data are expressed as means ±SD of three determinations.

* Cytochrome P450 was measured by the method of Omura and Sato [27].

† Cytochrome P450 was measured by the method of Estabrook *et al.* [28].

‡ Significantly lower ($P \leq 0.05$) from control (+NADPH) and incubation in the presence of the respective drug in the absence of NADPH, as determined by a repeated-measures design one-way analysis of variance and Newman–Keuls test.

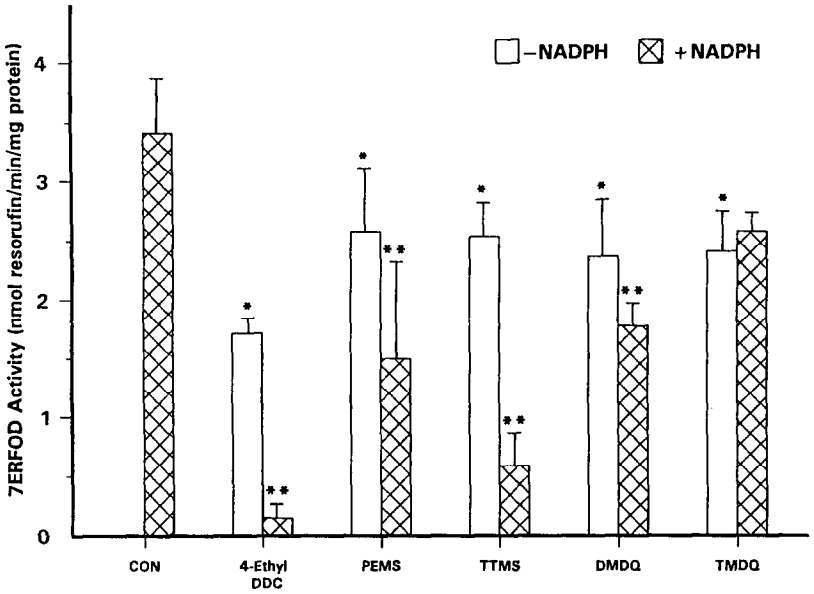

Fig. 2. Effect of 4-ethyl DDC (45 μM), the sydnones (250 μM), and the dihydroquinolines (100 μM) on 7ERFOD activity in hepatic microsomes from βNF-treated rats. Microsomes were incubated for 30 min with the respective drug in the presence or absence of NADPH, reharvested by ultracentrifugation, and then assayed for enzyme activity. Control (CON) represents initial incubation in the presence of NADPH alone. Each bar represents the mean (±SD) of determinations from three rats. Key: (*) significantly lower ($P \leq 0.05$) from control; and (**) significantly lower ($P \leq 0.05$) from control and incubation in the presence of the respective drug in the absence of NADPH, as determined by a repeated-measures design one-way analysis of variance and Newman–Keuls test.

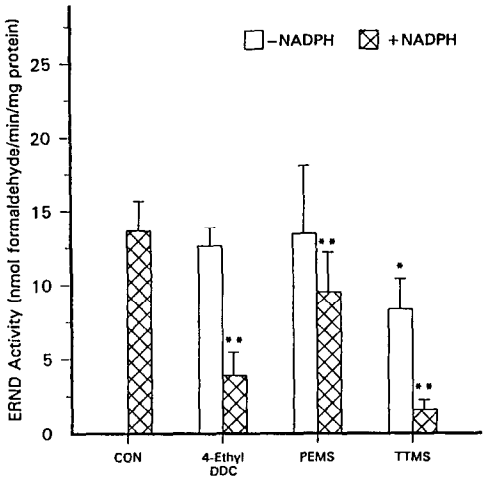

Fig. 3. Effect of 4-ethyl DDC (45 μM) and the sydnones (250 μM) on ERND activity in hepatic microsomes from DEX-treated rats. Microsomes were incubated for 30 min with the respective drug in the presence or absence of NADPH, reharvested by ultracentrifugation, and then assayed for enzyme activity. Control (CON) represents initial incubation in the presence of NADPH alone. Each bar represents the mean (± SD) of determinations from four rats. Key: (*) significantly lower ($P \leq 0.05$) from control; and (**) significantly lower ($P \leq 0.05$) from control and incubation in the presence of the respective drug in the absence of NADPH, as determined by a repeated-measures design one-way analysis of variance and Newman–Keuls test.

7ERFOD activity was likely due to competitive inhibition of P4501A by drugs that remained in the microsomes despite centrifugation.

Mechanism-based inactivation of P4503A in rat hepatic microsomes assessed using ERND activity as a catalytic marker. Following the incubation of microsomes with NADPH and a dihydropyridine, sydnone, or dihydroquinoline, the microsomes were harvested by ultracentrifugation (100,000 *g*) and assayed for functional activity. Mechanism-based inactivation of P4503A was examined by measuring ERND activity in DEX-induced rat hepatic microsomes (Fig. 3). 4-Ethyl DDC, PEMS, and TTMS caused a depression of ERND activity in the presence of NADPH that was significantly different from that measured in control (−drug, +NADPH) and that measured in the presence of drug but absence of NADPH. This NADPH-dependent inhibition shows that P4503A is a target for mechanism-based inactivation in the rat. DMDQ and TMDQ did not cause a significant depression of ERND activity (results not shown).

Determination of the optimal dosage regimen to induce maximal levels of chick embryo hepatic cytochrome P450. The optimal dose of PB, βNF, and DEX to induce maximal levels of hepatic cytochrome P450 in the rat is well established, i.e. 80 mg PB/kg/day for 4 days, 40 mg βNF/kg/day for 3 days, and 100 mg DEX/kg/day for 4 days [13]. The optimal dose of PB to induce maximal P450 in the chick embryo has been determined previously to be 6.0 mg/egg/day for 2 days [26]. However, we needed to establish the appropriate dose of βNF and DEX

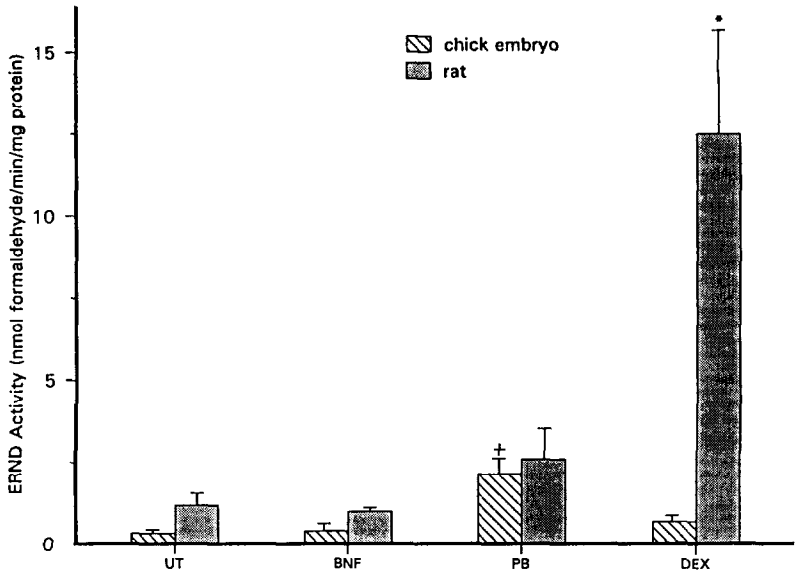

Fig. 4. Effect of induction by PB, βNF, and DEX on ERND activity in rat and chick embryo hepatic microsomes. Each bar represents the mean (±SD) of determinations from three experiments. The livers of twelve chick embryos were pooled to constitute one experiment. Key: (*) significantly different from untreated (UT), βNF-, and PB-treated rat hepatic microsomes; and (+) significantly different from untreated (UT), βNF-, and DEX-treated chick embryo hepatic microsomes, as determined by a repeated-measures design one-way analysis of variance and Newman–Keuls test.

in the chick embryo. βNF was therefore administered to the chick embryos in doses of 1.0, 2.0, and 4.0 mg/egg/day for 1, 2, and 3 days, and DEX was administered in doses of 2.5, 5.0, and 7.5 mg/egg/day for 1, 2, and 3 days, such that all the chick embryos were incubated for 19 days at the end of the treatment period. It was noted that DEX had a toxic effect on the chick embryos after 3 days of dosing. Induction of hepatic cytochrome P450 was maximal with a dose of 2.0 mg βNF/egg for 1 day, and 5.0 mg DEX/egg for 1 day (results not shown).

Effect of PB and βNF induction on 7ERFOD activity in chick embryo liver. The effect of induction by PB and βNF on 7ERFOD activity, a catalytic marker for P4501A in the rat, was examined in chick embryo liver microsomes (results not shown). In PB-induced microsomes, 7ERFOD activity was no different than in untreated microsomes (0.03 nmol resorufin/min/mg protein). In βNF-induced microsomes, 7ERFOD activity increased approximately 90-fold. In rats treated with βNF, P4501A1 represents nearly 45% of the total P450, as compared with 2% in the uninduced state [35]. Therefore, in both the rat and the chick embryo, βNF is an excellent inducer of P4501A isozymes.

Effects of PB, βNF, and DEX induction on ERND activity in rat and chick embryo liver. The effect of induction by PB, βNF, and DEX on ERND activity, a catalytic marker for P4503A1/2 in the rat, was examined in both rat and chick embryo liver microsomes. In the rat (Fig. 4), βNF-induced microsomes did not show increased ERND activity when compared with the uninduced state (1.18 nmol HCHO/min/mg protein). ERND activity in PB-induced microsomes was 2-fold greater than in uninduced microsomes, while activity in DEX-induced microsomes was more than 10-fold greater. In the chick embryo (Fig. 4), βNF-induced microsomes did not show increased ERND activity when compared with the uninduced state (0.32 nmol HCHO/min/mg protein). ERND activity in DEX-induced microsomes was 2-fold greater than in uninduced microsomes, whereas activity in PB-induced microsomes was more than 6-fold greater. Lorr *et al.* [19] also observed a greater increase in ERND activity with PB-induced than uninduced hepatic microsomes obtained from 14- or 18-day-old chick embryos, and greater ERND activity in hepatic microsomes obtained from PB-induced than DEX-induced or uninduced microsomes in chickens at 1 or 36 days posthatch. However, it was observed by Lorr *et al.* [19] and in the current study (results not shown) that DEX is a more specific inducer when the ERND activity is expressed as nanomoles of HCHO per nanomole P450 per minute. In the chick embryo, we chose to induce with PB rather than DEX in order to obtain higher levels of ERND activity, which facilitated its measurement following mechanism-based inactivation.

Effects of the dihydropyridines, sydnones, and dihydroquinolines on cytochrome P450 content of chick embryo hepatic microsomes. It has been shown previously that lipid peroxidation is not a problem in chick embryo hepatic microsomes [26], as it is in rat hepatic microsomes [13], and therefore in the current study the chick embryo P450 levels were measured by the method of Omura and Sato [27]. The P450 loss in microsomes from untreated, βNF-, PB-, and DEX-treated chick embryos is shown in Table 2. 4-Ethyl DDC, 4-isopropyl DDC, and TTMS caused

Table 2. Effects of the dihydropyridines, sydnones, and dihydroquinolines (45 μM) on cytochrome P450 content of hepatic microsomes from untreated, βNF-, PB-, and DEX-treated chick embryos

Drug	Cytochrome P450 content (nmol/mg protein)			
	Untreated	βNF-treated	PB-treated	DEX-treated
Control	0.29 ± 0.03	0.54 ± 0.11	1.65 ± 0.26	0.35 ± 0.06
Control + NADPH	0.25 ± 0.03	0.47 ± 0.12	1.56 ± 0.25	0.34 ± 0.06
DDC	0.32 ± 0.01	0.56 ± 0.10	1.71 ± 0.29	0.41 ± 0.06
DDC + NADPH	0.22 ± 0.02	0.44 ± 0.11	1.45 ± 0.19*	0.31 ± 0.06
4-Ethyl DDC	0.31 ± 0.02	0.56 ± 0.08	1.74 ± 0.29	0.38 ± 0.07
4-Ethyl DDC + NADPH	0.20 ± 0.02*	0.31 ± 0.03*	1.19 ± 0.18*	0.19 ± 0.11*
4-Isopropyl DDC	0.31 ± 0.03	0.51 ± 0.12	1.76 ± 0.32	0.39 ± 0.05
4-Isopropyl DDC + NADPH	0.18 ± 0.01*	0.26 ± 0.01*	1.18 ± 0.18*	0.19 ± 0.06*
TTMS	0.29 ± 0.03	0.52 ± 0.11	1.72 ± 0.30	0.38 ± 0.10
TTMS + NADPH	0.20 ± 0.02*	0.33 ± 0.08*	1.13 ± 0.23*	0.22 ± 0.04*
PEMS	0.31 ± 0.04	0.48 ± 0.07	1.74 ± 0.28	0.39 ± 0.08
PEMS + NADPH	0.22 ± 0.03	0.37 ± 0.13*	1.42 ± 0.23*	0.26 ± 0.06*
DMDQ	0.31 ± 0.02	0.56 ± 0.10	1.74 ± 0.27	0.39 ± 0.09
DMDQ + NADPH	0.21 ± 0.03	0.35 ± 0.05*	1.49 ± 0.23	0.29 ± 0.06
TMDQ	0.33 ± 0.04	0.55 ± 0.10	1.70 ± 0.27	0.42 ± 0.11
TMDQ + NADPH	0.23 ± 0.03	0.39 ± 0.14	1.59 ± 0.24	0.30 ± 0.07

P450 was measured by the method of Omura and Sato [27]. All data are expressed as means ± SD of four determinations.

* Significantly lower ($P \leq 0.05$) from control (+NADPH) and incubation in the presence of the respective drug in the absence of NADPH, as determined by a repeated-measures design one-way analysis of variance and Newman–Keuls test.

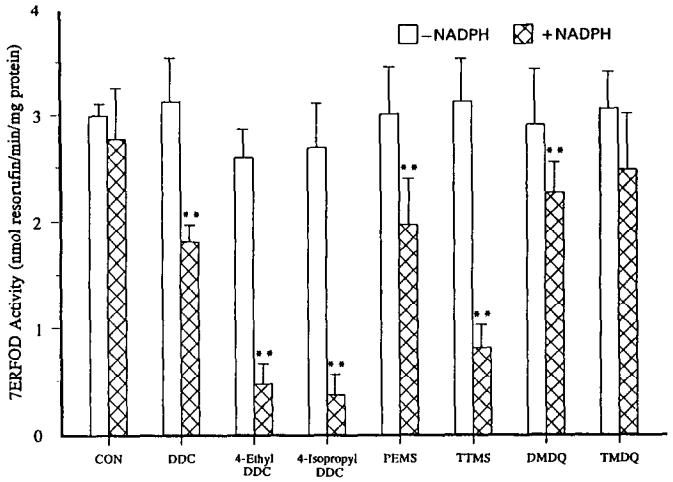

Fig. 5. Effect of the dihydropyridines, sydnones, and dihydroquinolines (45 μM) on 7ERFOD activity in hepatic microsomes from βNF-treated chick embryos. Microsomes were incubated for 30 min with the respective drug in the presence or absence of NADPH, reharvested by ultracentrifugation, and then assayed for enzyme activity. Each bar represents the mean (± SD) of determinations from four experiments. The livers of twelve chick embryos were pooled to constitute one experiment. Key: (**) significantly lower ($P \leq 0.05$) from control (CON) (−drug, +NADPH) and incubation in the presence of the respective drug in the absence of NADPH, as determined by a repeated-measures design one-way analysis of variance and Newman–Keuls test.

an NADPH-dependent loss of P450 in hepatic microsomes from untreated, βNF-, PB-, and DEX-treated chick embryos. PEMS caused an NADPH-dependent loss of P450 in βNF-, PB-, and DEX-treated microsomes, but not in untreated microsomes. DMDQ caused an NADPH-dependent loss of P450 in βNF-treated microsomes only, and DDC caused an NADPH-dependent loss of P450 in PB-treated microsomes only. TMDQ was inactive.

Mechanism-based inactivation of P4501A in chick

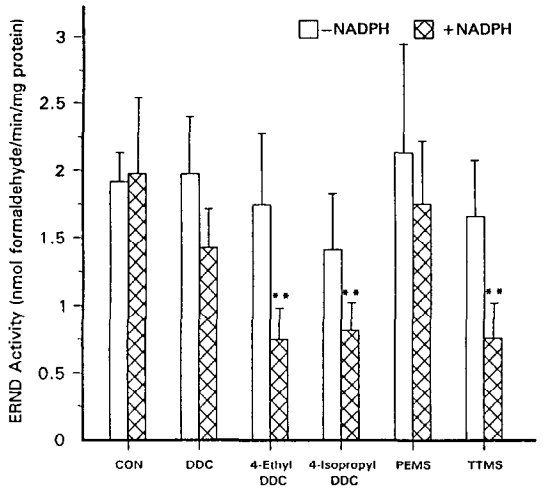

Fig. 6. Effect of the dihydropyridines and sydnones (45 μM) on ERND activity in hepatic microsomes from PB-treated chick embryos. Microsomes were incubated for 30 min with the respective drug in the presence or absence of NADPH, reharvested by ultracentrifugation, and then assayed for enzyme activity. Each bar represents the mean (± SD) of determinations from five experiments. The livers of twelve chick embryos were pooled to constitute one experiment. Key: (**) significantly lower ($P \leq 0.05$) from control (CON) (−drug, +NADPH) and incubation in the presence of the respective drug in the absence of NADPH, as determined by a repeated-measures design one-way analysis of variance and Newman–Keuls test.

embryo hepatic microsomes assessed using 7ERFOD activity as a catalytic marker. Following the incubation of microsomes with NADPH and a dihydropyridine, sydnone, or dihydroquinoline, the microsomes were harvested by ultracentrifugation (100,000 *g*) and assayed for functional activity. Mechanism-based inactivation of P4501A was examined by measuring 7ERFOD activity in βNF-induced chick embryo hepatic microsomes (Fig. 5). All the drugs tested, except TMDQ, caused a depression of 7ERFOD activity in the presence of NADPH that was significantly different from that measured in control (−drug, +NADPH) and that measured in the presence of drug but in the absence of NADPH. This NADPH-dependent inhibition shows that P4501A is a target for mechanism-based inactivation in the chick embryo liver.

Mechanism-based inactivation of P4503A in chick embryo hepatic microsomes assessed using ERND activity as a catalytic marker. Following the incubation of microsomes with NADPH and a dihydropyridine, sydnone, or dihydroquinoline, the microsomes were harvested by ultracentrifugation (100,000 *g*) and assayed for functional activity. Mechanism-based inactivation of P4503A was examined by measuring ERND activity in PB-induced chick embryo hepatic microsomes (Fig. 6). 4-Ethyl DDC, 4-isopropyl DDC, and TTMS caused a depression of ERND activity in the presence of NADPH that was significantly different from that measured in control (−drug, +NADPH) and that measured in the presence of drug but absence of NADPH. This NADPH-dependent inhibition suggests that P4503A may be a target for mechanism-based inactivation in the chick embryo. DDC and PEMS (Fig. 6), and DMDQ and TMDQ (results not shown), were inactive.

The first objective of our study was to determine whether rat hepatic P4501A and P4503A, targets for mechanism-based inactivation by dihydropyridines, were also targets for mechanism-based inactivation by sydnones and dihydroquinolines. The answer is: (1) that P4501A is a target for both sydnones, TTMS and PEMS, but is a target for only the more potent of the two dihydroquinolines, namely, DMDQ, and (2) that P4503A is a target for the sydnones, TTMS and PEMS, but not for the dihydroquinolines. The latter result agrees with a previous report [9] which failed to demonstrate mechanism-based inactivation of P4503A1/2, 2B1, or 2C11 by DMDQ.

The second objective of our study was to determine whether P4501A and 3A of chick embryo liver were targets for mechanism-based inactivation by dihydropyridines, as had been shown to be the case with rat liver P4501A1 and 3A1/2 isozymes [13], and whether they were also targets for the sydnones and dihydroquinolines. The answer is that P4501A and 3A of chick embryo liver share the ability of the rat P4501A1 and 3A1/2 isozymes to serve as targets for inactivation by 4-ethyl DDC (Figs. 2 and 3) and 4-isopropyl DDC [13]. On the other hand, the results with DDC where mechanism-based inactivation was observed in chick embryo liver, differ from results obtained in the βNF-treated rat [13] where no effect of DDC on 7ERFOD activity was observed in rat hepatic microsomes, and it was concluded that P4501A1 is not a target for inactivation by DDC. There are two possible explanations for this difference in action of DDC in the chick embryo and the rat: (1) 7ERFOD activity is a catalytic marker in chick embryo hepatic microsomes for both P4501A and a related P450 isozyme(s) with the capacity to undergo mechanism-based inactivation by DDC, and (2) it is known that a single change in an amino acid of a P450 isozyme can result in a marked change in enzymic activity [36], and such a small difference between the P4501A of rat liver and a similar isozyme in chick embryo liver may result in a modified substrate specificity. The sydnones, TTMS and PEMS, and the dihydroquinoline, DMDQ, functioned as mechanism-based inactivators of chick embryo liver P4501A (Fig. 5), results that parallel their effect on the rat P4501A isozyme(s) (Fig. 2). The sydnone TTMS functioned as a mechanism-based inactivator of chick embryo liver P4503A (Fig. 6), a result that parallelled results on the rat P4503A isozyme(s) (Fig. 3). However, PEMS, which was active on the rat isozyme(s) (Fig. 3), was inactive on the chick embryo isozyme(s) (Fig. 6). The dihydroquinolines were devoid of activity on either the rat or chick embryo P4503A isozyme(s).

There is considerable interest in the development of mechanism-based inactivators selective for P450 isozymes in various tissues [37]. It is therefore of interest that DDC is selective for the P4501A isozyme(s) of chick embryo liver versus rat liver P4501A1, and that PEMS is selective for the P4503A

isozyme(s) of rat liver versus chick embryo liver P4503A.

In conclusion, the data show a remarkable similarity between the mechanism-based inactivation observed with the dihydropyridines, sydnones, and dihydroquinolines in P4501A and P4503A isozymes in chick embryo and rat liver. The similar results obtained reinforces the view that in the chick embryo liver we are dealing with the chicken equivalents of rat hepatic P4501A and 3A subfamilies.

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