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Effects of a Series of 4-Alkyl Analogues of 3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine on the Major Inducible Cytochrome P-450 Isozymes of Rat Liver

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SUMMARY

Various 4-alkyl analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) cause mechanism-based inactivation of cytochrome P-450 (P-450) by destroying the heme prosthetic group. We have examined the isozyme selectivity of representative DDC analogues with respect to the major inducible P-450 isozymes of rat liver. Hepatic microsomes from untreated, phenobarbital (PB)-treated, β-naphthoflavone (βNF)-treated, and dexamethasone (DEX)-treated rats were incubated with a DDC analogue and NADPH and were subsequently analyzed for P-450 and heme content, P-450 isozyme immunoreactivity, and enzyme activity. Compared with the uninduced state, 4-isopropyl-DDC caused slightly less P-450 destruction following βNF induction and much greater destruction following DEX pretreatment. Also, 4-hexyl-DDC was found to cause less P-450 destruction following PB or DEX pretreatment, compared with results obtained with untreated rats. These results suggest that DDC analogues possess different isozyme selectivity profiles. Monoclonal antibodies (MAbs) directed against the major inducible isozymes of P-450 were used to probe Western blots of microsomal protein following DDC analogue treatment. The formation of lower molecular mass (45–55 kDa) immunoreactive proteins in microsomes from βNF-treated rats following DDC analogue treatment was revealed by two MAbs (1-31-2 and 1-36-1), suggesting that the apoprotein of the major βNF-inducible isozyme, P-450c, is subject to alteration by DDC analogues. In microsomes from DEX-treated rats, DDC analogues caused the formation of higher molecular mass (80, 94, and 115 kDa) proteins showing immunoreactivity with MAb 2-13-1, directed against a major DEX-inducible isozyme belonging to the P-450p family. These immunochemical findings are supported by the demonstration that DDC analogues also caused mechanism-based inhibition of the catalytic activity of P-450c (7-ethoxyresorufin *O*-deethylase) and P-450p (erythromycin *N*-demethylase) but not that of the major PB-inducible isozyme, P-450b (7-pentoxyresorufin *O*-dealkylase). The combined immunochemical and enzymic studies indicate that rat liver P-450 c and p are targets for mechanism-based inactivation by DDC analogues.

The dihydropyridine DDC (Fig. 1, compound **a**) and various 4-alkyl analogues (Fig. 1, compounds **b–e**) are porphyrinogenic compounds that are valuable in the study of the regulation of heme biosynthesis (1). DDC analogues cause mechanism-based or suicidal inactivation of P-450 (2–4). Current ideas suggest that a radical cation is formed by the one-electron oxidation of the DDC analogue's nitrogen atom by P-450, and this is followed by ejection of the 4-alkyl radical, which, in most cases, alkylates one of the four pyrrole nitrogens of the P-450 heme prosthetic group (2). The result is the production of the four regioisomers of the corresponding *N*-alkylPP, which differ with respect to the pyrrole ring (A, B, C, or D) nitrogen that is alkylated (5–7). The *N*-alkylPP thus formed inhibits ferrochelatase (EC 4.99.1.1) (3, 4, 7), resulting in the accumulation of protoporphyrin IX. In the case of *N*-alkylPPs with an *N*-alkyl group other than methyl, the ferrochelatase-inhibitory activity resides preferentially in the N_A and N_B regioisomers (1, 7). With various DDC analogues, the fate of the P-450 heme and the regioselectivity of heme alkylation differ (5–7), thus affecting the ability of these compounds to inhibit ferrochelatase (7). Recently, it has become apparent that suicide substrates of P-450 such as 4-ethyl-DDC (Fig. 1, compound **b**) and AIA not only cause formation of *N*-alkylPPs but also activate the prosthetic heme to products that bind irreversibly to the apoprotein (8, 9).

DDC analogues that inactivate P-450 can be classified into three groups. The first group, typified by 4-ethyl-DDC (Fig. 1, compound **b**) and 4-hexyl-DDC (Fig. 1, compound **e**), destroys

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ABBREVIATIONS: DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; P-450, cytochrome P-450; PB, phenobarbital; βNF, β-naphthoflavone; DEX, dexamethasone; MAb, monoclonal antibody; 7ERFOD, 7-ethoxyresorufin *O*-deethylase; ERND, erythromycin *N*-demethylase; 7PRFOD, 7-pentoxyresorufin *O*-dealkylase; *N*-alkylPP, *N*-alkylprotoporphyrin IX; AIA, allylisopropylacetamide; MC, 3-methylcholanthrene; PCN, pregnenolone 16α-carbonitrile; TBS, Tris-buffered saline; BSA, bovine serum albumin.

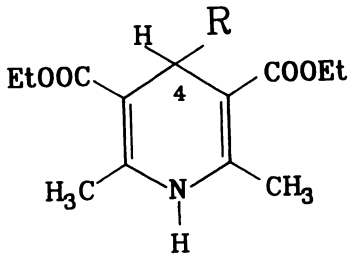

Fig. 1. Structure of dihydropyridine analogues. **a**, R = —CH_3, DDC; **b**, R = —CH_2CH_3, 4-ethyl-DDC; **c**, R = —$CH(CH_3)_2$, 4-isopropyl-DDC; **d**, R = —$CH_2CH(CH_3)_2$, 4-isobutyl-DDC; **e**, R = —$(CH_2)_5CH_3$, 4-hexyl-DDC.

P-450 heme, leading to the formation of an *N*-alkylPP that inhibits ferrochelatase. The second group, typified by 4-isopropyl-DDC (Fig. 1, compound **c**), destroys P-450 heme, but the heme is believed to be degraded to products other than an *N*-alkylPP (2) and, hence ferrochelatase is not inhibited. The third group, typified by 4-isobutyl-DDC (Fig. 1, compound **d**), destroys P-450 heme, leading to the formation of an *N*-alkylPP; however, because of the regioisomer composition of the resulting *N*-alkylPP, this compound is essentially devoid of ferrochelatase-inhibitory activity (4, 7).

Multiple isozymes of P-450 exist (10), making it difficult to define the role that individual forms play in biotransformation. Isozymes that are inducible by xenobiotics are particularly amenable to study. Polycyclic aromatic hydrocarbons such as βNF and MC induce members of the P-450IA subfamily (P-450 c and d),[2] PB induces isozymes in the P-450IIB subfamily (P-450 b and e), and steroids such as DEX and PCN induce P-450IIIA subfamily isozymes (P-450p and related forms).

In order to clarify the porphyrinogenic mechanisms of the DDC analogues, we wish to determine whether the interaction of DDC analogues with specific P-450 isozymes determines the regioisomer composition of the resulting *N*-alkylPP and, hence, determines the ferrochelatase-lowering activity and porphyrinogenicity of these compounds. Few studies have examined the isozyme selectivity of DDC analogues. Recently, Tephly *et al.* (19) have shown that the administration of 4-ethyl-DDC to rats leads to a loss of the immunoreactive protein moiety of P-450 isozymes c, h, k, and p. This was the first demonstration of such a loss of the P-450 apoprotein caused by a DDC analogue. In addition, Correia *et al.* (20) demonstrated that administration of 4-ethyl-DDC to rats results in the loss of catalytic markers selective for isozymes h, k, and p. In particular, this group has identified the steroid-inducible P-450p isozyme as a key target for mechanism-based inactivation by 4-ethyl-DDC. Heme-protein adduct formation, as opposed to *N*-alkylation, appears to be the major fate of the prosthetic heme of this isozyme (20).

The goal of the present work was to determine the effect of DDC analogues on the catalytic activities and apoprotein moieties of the three major families of inducible P-450 isozymes in an *in vitro* microsomal preparation. The major isozymes induced by PB, βNF, and DEX are referred to as P-450 b, c, and p, respectively.

Materials and Methods

Source of compounds. DDC analogues were synthesized as described previously (2, 4). MAbs were prepared by the hybridoma method described previously (21–23). AIA was obtained as a gift from Hoffman-LaRoche, Ltd. (Vaudreil, Quebec), and 1-aminobenzotriazole was provided by Dr. P. R. Ortiz de Montellano (Department of Pharmaceutical Chemistry, University of California, San Francisco, CA). Chemicals were purchased from the following sources: sodium PB, Allen & Hanburys (Toronto, Ontario); βNF Aldrich Chemical Co. (Milwaukee WI); resorufin, 7-ethoxyresorufin, and 7-pentoxyresorufin, Molecular Probes, Inc. (Eugene, OR); DEX, NADPH, and erythromycin, Sigma Chemical Co. (St. Louis, MO); and molecular weight standards, Pharmacia Fine Chemicals (Piscataway, NJ). All other chemicals for electrophoresis, blotting, and immunodetection were purchased from Bio-Rad Laboratories (Richmond, CA).

Animals and treatment. Male Sprague-Dawley rats (250–300 g) were obtained from Charles River Canada, Inc. (St.-Constant, Quebec). The rats were fed Purina Lab Chow and water *ad libitum* and were housed under controlled conditions (22°, 12-hr light/12-hr dark cycle). Rats either received no treatment or were injected intraperitoneally with PB (80 mg/kg in water daily for 4 days), βNF (40 mg/kg in corn oil daily for 3 days), or DEX (100 mg/kg in corn oil daily for 4 days).

Preparation and storage of hepatic microsomes. Twenty-four hours after the last treatment, animals were sacrificed by decapitation. Livers were perfused *in situ* with ice-cold 1.15% KCl, removed, weighed, and homogenized in 4 volumes of cold phosphate-buffered KCl (1.15% KCl, 10 mM K_2HPO_4, pH 7.4). Microsomes were isolated by differential centrifugation, as described previously (3). The final microsomal pellet was either used immediately or frozen in polypropylene ultracentrifuge tubes by immersion in liquid nitrogen. These microsomes were stored at −70° for periods of up to 2 weeks, with no loss of P-450 and heme content (data not shown).

Inactivation of microsomal P-450 and heme. The general procedure has been described previously (3). Microsomal pellets corresponding to 1 g of liver were resuspended in a total volume of 5 ml of 0.1 M K_2HPO_4 buffer (pH 7.4) containing 1.5 mM EDTA. The microsomal suspension was diluted 2-fold with the above incubation buffer and a DDC analogue in 95% ethanol (10 μl) was added to this suspension (4 ml) to yield a final concentration of 0.45 mM. The microsomal suspension was incubated in the presence of the DDC analogue and 1.0 mM NADPH for 30 min at 37° in a shaking water bath. Conditions were designed to maximize P-450 destruction. When enzyme activities were to be measured, a 10-fold lower dose of the DDC analogues (i.e., 45 μM) was used to reduce the effects of residual compound remaining in the microsomes. In all cases, this dose of DDC analogue caused significant P-450 and heme destruction. In all experiments, the following controls were run concurrently: (a) omission of NADPH and (b) omission of the DDC analogue. Reactions were terminated by cooling samples on ice, and P-450 and heme levels were determined spectrophotometrically by the methods of Omura and Sato (24), as described previously (3). Under the experimental conditions employed, P-450 content determined by this method did not differ significantly from that determined by the method of Estabrook *et al.* (25). Protein was assayed by the method of Lowry *et al.* (26). The susceptibility of microsomal preparations to lipid peroxidation during this *in vitro* incubation was assessed by measuring malondialdehyde formation by the method of Buege and Aust (27). Determinations were made following incubation in the presence of 1.0 mM NADPH and 1.5 mM EDTA for 0 and 30 min.

Immunodetection of P-450 isozymes. Samples from each of the

[2] Designations given to apparently equivalent P-450 preparations include the following: P-450a = UT-F, 3 (gene IIA1); P-450b = PB-B, PB-4 (gene IIB1); P-450c = βNF-B (gene IA1); P-450d = ISF-G (gene IA2); P-450e = PB-D, PB-5 (gene IIB2); P-450h = UT-A, 2c, RLM5, male (gene IIC11); P-450k = PB-C, PB-1 (gene IIC6) (10–12). At present, there is confusion in the literature concerning the nomenclature of members of the steroid-inducible IIIA subfamily. It has been established that the PCN-inducible isozyme P-450p (13) is equivalent to PCN1 (14) and PCNa (15) and this isozyme has the gene designation IIIA1 (11). It appears that a second PCN-inducible isozyme exists, which has been referred to as PB/PCN-E (16), PB-2a (17), and PCNb (15). In addition, closely related constitutive male forms such as PCN2 (14), PCNc (18), and 2a (12) have been reported. For the purpose of this manuscript, the term P-450p is used in a general sense to refer to closely related isozymes of the steroid-inducible P-450 IIIA subfamily.

reaction mixtures described above were solubilized by boiling at 100° for 5 min in a solution containing 62.5 mM Tris·HCl (pH 6.8), 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue. Microsomal proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 7.5% (w/v) acrylamide resolving gel, by the method of Laemmli (28). The separated proteins were electrophoretically transferred to nitrocellulose sheets (0.45 μm) by the method of Towbin *et al.* (29). After transfer, strips of nitrocellulose were stained with Coomassie brilliant blue R-250 to identify molecular weight standards, and the gels were silver stained to assess transfer efficiency.

For immunodetection of P-450 isozymes immobilized on nitrocellulose, nonspecific sites were blocked by incubating sheets in TBS (0.02 M Tris·HCl, 0.5 M NaCl, pH 7.5) that contained 3% BSA, for 40 min, followed by TBS that contained 0.05% Tween-20, for 20 min. Sheets were incubated overnight at 4° in the presence of the primary MAb (mouse anti-rat P-450) diluted with TBS that contained 1% BSA. MAbs directed toward MC-inducible rat liver P-450, MC-P-450 (1-31-2, 1-36-1), were used at a 1:100 dilution, whereas MAbs directed toward PB-inducible rat liver P-450, PB-P-450 (2-8-1, 2-66-3), were used at a 1:40 dilution. MAb 2-13-1, directed against the PCN-inducible isozyme, P-4502a/PCN-E, was used at a 1:1500 dilution. MAb HyHel-9, directed toward chicken lysozyme, was used as a primary antibody to control for nonspecific binding in Western blots. These MAbs have been characterized previously (21–23). After washing in TBS, sheets were exposed to a goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate, at a 1:2000 dilution in TBS that contained 1% BSA, for 1 hr at room temperature. After further washing in TBS, a brown color developed upon the addition of 2.3 mM 3,3′-diaminobenzidine and 0.015% H_2O_2 in TBS. The relative quantities of immunostained P-450 isozymes in control and DDC analogue-treated microsomal preparations were compared by laser densitometry using an LKB 2202 Ultroscan laser densitometer.

Enzyme assays. Following the inactivation of microsomal P-450 by DDC analogues (45 μM), microsomes were reharvested by centrifugation for 1 hr at 106,000 × *g*. Such microsomes were then monitored for catalytic function using three isozyme-selective enzyme assays.

Microsomal pellets from PB- and βNF-treated rats were resuspended in 0.1 M Na/K-PO_4 buffer (pH 7.6) to give 0.1–0.2 mg of protein/ml and were assayed for 7PRFOD and 7ERFOD activities, respectively, according to the method of Burke *et al.* (30). Microsomes were incubated for 2 min at 37° in the presence of 5.0 μM of the appropriate substrate (15 μl of a 1.0 mM stock in dimethylsulfoxide added to a 3.0-ml reaction mixture). The reaction was initiated by the addition of NADPH (final concentration of 0.25 mM) and the formation of the product, resorufin, was monitored fluorometrically over time using a Perkin-Elmer LS-5B luminescence spectrometer (excitation and emission wavelengths, 530 and 585 nm, respectively; slits, 5 nm). The linear portion of the progress curve was used to estimate initial reaction velocity. Resorufin formation was quantitated by comparison with a standard calibration curve relating fluorescence intensity to resorufin concentration (0–0.2 nmol/ml). For resorufin standards, fluorescence was always measured in the presence of 5.0 μM 7-ethoxyresorufin or 7-pentoxyresorufin in order to mimic experimental conditions. Reaction conditions were selected to achieve optimal enzyme activity, and reaction velocity was directly proportional to microsomal protein content over the range 0–0.75 mg/ml. In a separate experiment, we examined the direct inhibitory effects of 4-ethyl-DDC on 7PRFOD activity in the absence of a 30-min preincubation. For this purpose, microsomes from PB-treated rats were suspended in 0.1 M Na/K-PO_4 buffer (pH 7.6) to give a protein content of approximately 0.35 mg/ml. Enzyme activity was measured as described above, with substrate concentrations of 0, 0.5, 1.0, 2.5, and 5.0 μM, in the absence and presence of 4-ethyl-DDC (10^{-8} or 10^{-6} M). Incubations were run to control for effects of the substrate vehicle (dimethylsulfoxide) and the inhibitor vehicle (95% ethanol).

Following the 30-min preincubation and centrifugation described above, microsomal pellets from DEX-treated rats were resuspended in 0.1 M K_2HPO_4 buffer (pH 7.4), to yield a final protein content of 0.8–1.0 mg/ml, and were assayed for ERND activity according to the method of Wrighton *et al.* (31). Incubations were run at 37° in the presence of 1.0 mM erythromycin and 1.2 mM NADPH for 10 min and then analyzed for formaldehyde (HCHO) content. Product formation was linear with respect to incubation time (0–20 min) and protein content (0–3.0 mg/ml).

Statistical analysis. For comparison of independent treatments with control, a randomized design one-way analysis of variance was employed. For comparison of treatments and control measurements derived from the same animal, a repeated measures design one-way analysis of variance was used. In both cases, significantly different groups were identified using a Newman-Kuels test ($p \leq 0.05$ or 0.01).

Results

In order to examine the isozyme selectivity of DDC analogues, five representative compounds were selected for study. DDC, 4-ethyl-DDC, and 4-hexyl-DDC are representatives of the first group, which cause P-450 destruction, *N*-alkylPP formation, and ferrochelatase inhibition. 4-Isopropyl-DDC is a representative of the second group, which destroys P-450 heme but does not form an *N*-alkylPP. Finally, 4-isobutyl DDC is representative of the third group, which also destroys P-450 heme and forms an *N*-alkylPP and yet does not inhibit ferrochelatase.

Effect of P-450 inducers on P-450 and heme loss caused by DDC analogues. With the exception of DDC, all compounds tested caused an NADPH-dependent loss of P-450 and heme in microsomes derived from untreated, PB-treated, βNF-treated, and DEX-treated rats (Table 1). The inability of DDC to cause significant loss of P-450 in rat liver microsomes has been reported previously (2) and contrasts with its ability to destroy P-450 in chick embryo hepatic microsomes (3). Examination of the data in Table 1 reveals that, in microsomes from DEX-treated rats, P-450 and heme levels are significantly higher following incubation in the presence of a DDC analogue alone, as compared with incubation in the presence of NADPH alone. This observation suggested that the process of NADPH-dependent lipid peroxidation was occurring in these microsomes despite the inclusion of 1.5 mM EDTA. Indeed, when we examined the susceptibility of our microsomal preparations to lipid peroxidation, it was demonstrated that only microsomes from DEX-treated rats generated significant amounts of malondialdehyde during a 30-min incubation in the presence of 1.0 mM NADPH (Table 2). Such lipid peroxidation appears to result in loss of approximately 20% of the total P-450 in these microsomes. For this reason, effects of DDC analogues are expressed with respect to the control incubation in the presence of NADPH alone.

Because basal levels of P-450 differ in microsomes from untreated, PB-treated, βNF-treated, and DEX-treated rats, it is useful to compare the P-450 loss caused by DDC analogues as a percentage of the basal level in the four microsomal preparations (Fig. 2). In the case of 4-ethyl-DDC and 4-isobutyl-DDC, induction with PB, βNF, or DEX has no significant effect on the fractional degree of P-450 destruction. However, with 4-isopropyl-DDC, the per cent loss of P-450 was found to be less in microsomes from βNF-treated rats, compared with the uninduced condition. Pretreatment with DEX resulted in a large increase in P-450 destruction caused by 4-isopropyl-DDC, compared with the uninduced, PB-induced, or βNF-

TABLE 1

Effect of DDC analogues on cytochrome P-450 and heme content of hepatic microsomes from untreated, PB-treated, βNF-treated, and DEX-treated rats

All data are expressed in units of nmol/mg of protein, as means (± standard deviation) of determinations from the number of rats indicated in parentheses.

Condition	Untreated		PB-treated		βNF-treated		DEX-treated	
	P-450 (5)	Heme (5)	P-450 (5)	Heme (5)	P-450 (6)	Heme (6)	P-450 (10)	Heme (7)
	nmol/mg of protein							
NADPH	0.96 ± 0.19	2.06 ± 0.21	1.51 ± 0.05	2.97 ± 0.07	1.15 ± 0.16	2.25 ± 0.18	1.70 ± 0.19	2.91 ± 0.31
DDC	0.94 ± 0.14	2.21 ± 0.16	1.48 ± 0.17	3.23 ± 0.55	1.24 ± 0.21	2.27 ± 0.26	2.04 ± 0.17*	3.15 ± 0.31*
DDC + NADPH	0.84 ± 0.20	2.07 ± 0.23	1.38 ± 0.03	2.83 ± 0.26	1.16 ± 0.19	2.32 ± 0.19	1.78 ± 0.18	2.80 ± 0.41
Ethyl	0.87 ± 0.11	2.21 ± 0.17	1.47 ± 0.09	3.14 ± 0.20	1.17 ± 0.20	2.32 ± 0.17	2.08 ± 0.16*	3.22 ± 0.37*
Ethyl + NADPH	0.46 ± 0.09*	1.59 ± 0.14*	0.92 ± 0.14*	2.01 ± 0.32*	0.67 ± 0.09*	1.80 ± 0.21*	0.86 ± 0.14*	1.72 ± 0.32*
Isopropyl	0.84 ± 0.11	2.27 ± 0.37	1.48 ± 0.08	2.98 ± 0.29	1.18 ± 0.16	2.32 ± 0.23	2.04 ± 0.16*	3.21 ± 0.40*
Isopropyl + NADPH	0.41 ± 0.09*	1.44 ± 0.11*	0.68 ± 0.17*	1.87 ± 0.19*	0.62 ± 0.08*	1.81 ± 0.14*	0.45 ± 0.07*	1.37 ± 0.26*
Isobutyl	0.83 ± 0.05	2.13 ± 0.16	1.49 ± 0.12	3.04 ± 0.20	1.10 ± 0.16	2.30 ± 0.20	2.00 ± 0.18*	3.14 ± 0.34*
Isobutyl + NADPH	0.53 ± 0.07*	1.61 ± 0.10*	0.98 ± 0.16*	2.47 ± 0.45*	0.67 ± 0.06*	1.86 ± 0.18*	0.78 ± 0.13*	1.74 ± 0.46*
Hexyl	0.89 ± 0.06	2.05 ± 0.20	1.53 ± 0.11	3.00 ± 0.25	1.17 ± 0.16	2.26 ± 0.17	2.02 ± 0.14*	3.14 ± 0.37*
Hexyl + NADPH	0.46 ± 0.05*	1.50 ± 0.16*	1.10 ± 0.16*	2.38 ± 0.48*	0.68 ± 0.13*	1.83 ± 0.16*	1.28 ± 0.19*	2.18 ± 0.38*

* Significantly different ($p \leq 0.01$) from incubation in the presence of NADPH alone, based on repeated measures design one-way analysis of variance and Newman-Keuls test.

TABLE 2

Susceptibility of rat hepatic microsomal preparations to lipid peroxidation during incubation at 37° in the presence of 1.5 mM EDTA and 1.0 mM NADPH

Data are expressed as means (± standard deviation) of four determinations.

Rat pretreatment	Incubation time	Malondialdehyde
	min	*nmol/mg of protein*
None	0	2.86 ± 0.68
	30	2.94 ± 0.23
PB	0	1.53 ± 0.33
	30	1.15 ± 0.14
βNF	0	2.36 ± 0.23
	30	1.87 ± 0.33
DEX	0	2.14 ± 0.36
	30	3.42 ± 0.15*

* Significantly different ($p \leq 0.01$) from 0-min incubation, based on randomized design one-way analysis of variance and Newman-Keuls test.

induced state. On the other hand, 4-hexyl-DDC destroyed a markedly lower percentage of P-450 in microsomes from PB-treated and DEX-treated rats, compared with that seen in microsomes from untreated rats. As a positive control for these studies, two mechanism-based inactivators of known isozyme selectivity were studied under identical conditions. As shown previously (32), AIA caused greatest P-450 inactivation in microsomes from PB-treated rats, whereas 1-aminobenzotriazole caused similar degrees of P-450 loss in microsomes from untreated, PB-treated, and βNF-treated rats (data not shown).

Effect of DDC analogues on P-450 isozyme immunoreactivity. Following incubation of microsomes with NADPH and a DDC analogue, the technique of Western blotting was used to assess the level of immunoreactive apoprotein of the major inducible isozymes of P-450. In all cases, microsomal samples derived from a single rat were run concurrently in adjacent wells on a single gel in order to facilitate comparison by densitometry. All experiments were repeated using microsomes from three or four rats and statistical analyses were performed on the densitometric data. Conditions employed in these experiments yielded a linear relationship between microsomal P-450 content (0–25 pmol) and the densitometric response for each MAb. The control MAb, HyHel-9, demonstrated no specific interaction with microsomes from untreated, PB-treated, βNF-treated, or DEX-treated rats (Fig. 3A). Two

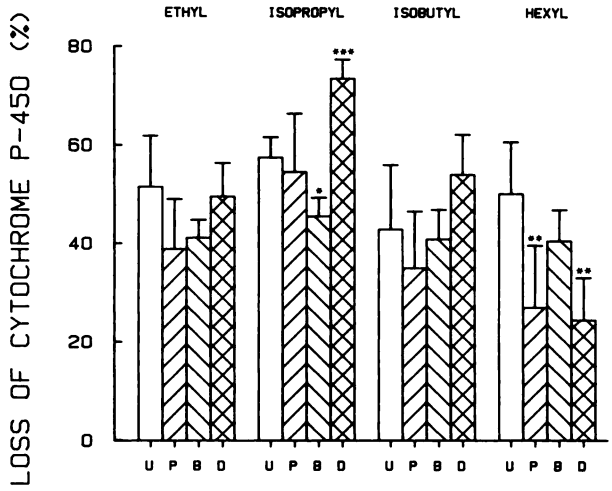

Fig. 2. Per cent loss of cytochrome P-450 caused by DDC analogues in hepatic microsomes from untreated (*U*), PB-treated (*P*), βNF-treated (*B*), and DEX-treated (*D*) rats. Loss of P-450 caused by incubation of microsomes in the presence of a DDC analogue and NADPH was expressed as a percentage of P-450 levels in microsomes incubated in the presence of NADPH alone. Each *bar* represents the mean (± standard deviation) of determinations from 5 (U and P), 6 (B), or 10 (D) rats. *Significantly different ($p \leq 0.01$) from P-450 loss observed in microsomes from untreated and DEX-treated rats; **significantly different ($p \leq 0.01$) from P-450 loss observed in microsomes from untreated rats; ***significantly different ($p \leq 0.01$) from P-450 loss observed in microsomes from untreated, PB-treated, and βNF-treated rats, as determined by a randomized design one-way analysis of variance and Newman-Kuels test.

MAbs, 1-31-2 and 1-36-1, recognize a single protein band of molecular mass 57 kDa in microsomes from βNF-treated rats, believed to be P-450c (12). After incubation of microsomes from βNF-treated rats with NADPH and a DDC analogue, MAbs 1-31-2 (Fig. 3B) and 1-36-1 (Fig. 3C) demonstrated the presence of lower molecular mass (45–55 kDa) immunoreactive proteins. These bands were significantly increased, compared with their levels in microsomal samples incubated in the presence of NADPH alone (Fig. 3, B and C, *lane 1*). Despite this significant accumulation of lower mass proteins after DDC analogue treatment, the main band recognized by these MAbs

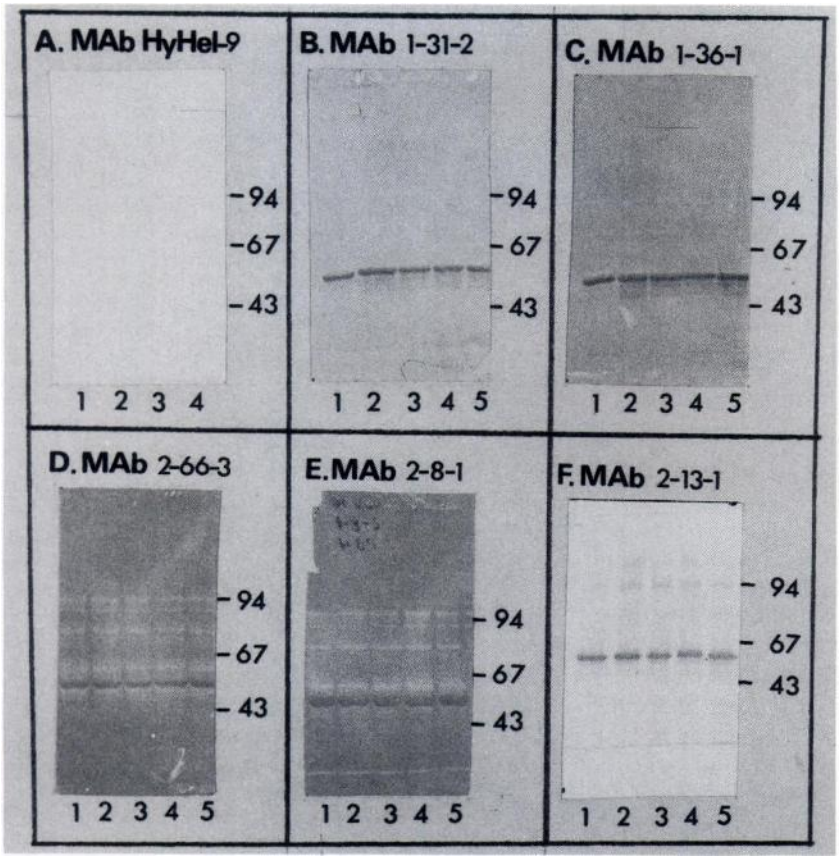

Fig. 3. Western blots of rat hepatic microsomal protein. Gel slots were loaded with 1–8 μg of microsomal protein. *Numbers* indicate distance of migration of molecular mass standards; 94 kDa, phosphorylase a; 67 kDa, BSA; 43 kDa, ovalbumin. A, Lack of specific staining observed with MAb HyHel-9 with microsomes from untreated (*lane 1*), PB-treated (*lane 2*), βNF-treated (*lane 3*), and DEX-treated (*lane 4*) rats. B, MAb 1-31-2 immunoreactivity in microsomes from a βNF-treated rat, incubated in the presence of NADPH alone (*lane 1*), or NADPH plus 4-ethyl-DDC (*lane 2*), 4-isopropyl-DDC (*lane 3*), 4-isobutyl-DDC (*lane 4*), and 4-hexyl-DDC (*lane 5*). C, MAb 1-36-1 immunoreactivity in microsomes from a βNF-treated rat. *Lanes* represent same treatments as in B. D, MAb 2-66-3 immunoreactivity in microsomes from a PB-treated rat. *Lanes* represent same treatments as in B. E, MAb 2-8-1 immunoreactivity in microsomes from a PB-treated rat. *Lanes* represent same treatments as in B. F, MAb 2-13-1 immunoreactivity in microsomes from a DEX-treated rat. *Lanes* represent same treatments as in B.

showed no alteration in immunoreactivity, even though the amount of microsomal protein used was always within the linear range of densitometric response.

MAbs 2-66-3 and 2-8-1, developed against the major PB-inducible isozyme of P-450, recognize a broad diffuse protein band of molecular mass 52–54 kDa in microsomes from PB-treated rats. These MAbs show highest reactivity with P-450b but also cross-react with other PB-inducible isozymes such as P-450 e and k, as well as P-450a (12). When microsomes from PB-treated rats were treated with NADPH and a DDC analogue, no alteration in MAb 2-66-3 (Fig. 3D) or 2-8-1 (Fig. 3E) immunoreactivity was detected.

MAb 2-13-1 recognizes a single protein band of molecular mass 54 kDa that is believed to represent the DEX-inducible PB-2a/PCN-E isozyme (12). This MAb also demonstrates cross-reactivity with the closely related P-450 2a form, a constitutive male-specific isozyme (12). It is not clear which, if any, other isozymes of the steroid-inducible (IIIA) subfamily are recognized by this MAb. We have used MAb 2-13-1 as a probe for immunoreactive protein moieties representative of the P-450p-related family of isozymes. Consistent with reports of others concerning the properties of PCN-inducible isozymes (31), the epitope recognized by MAb 2-13-1 was detected in microsomes from untreated and βNF-treated rats and was induced slightly by PB pretreatment (data not shown). The immunoreactivity was enhanced severalfold by DEX pretreatment. When microsomes from DEX-treated rats were incubated in the presence of a DDC analogue and NADPH, higher molecular mass (80, 94, and 115 kDa) immunoreactive proteins were detected at significantly higher levels than those in the control incubation in the presence of NADPH alone (Fig. 3F). Once again, this altered pattern of immunoreactivity was seen under conditions in which the main band recognized by MAb 2-13-1 did not change in density.

Effect of DDC analogues on microsomal enzyme activities. Following incubation of microsomes with NADPH and a DDC analogue, microsomes were reharvested by ultracentrifugation and assayed for functional activities. In initial experiments, DDC analogues were used at a concentration of 0.45 mM to cause maximal P-450 destruction. However, after centrifugation, residual DDC analogue apparently remained in the microsomes, making it difficult to assess contributions from mechanism-based enzyme inactivation and simple enzyme inhibition. This problem was largely eliminated by employing a DDC analogue dose of 45 μM in the initial preincubation. Three enzyme assays have been used to selectively probe the catalytic activity of the major inducible isozymes.

First, 7ERFOD activity in microsomes from βNF-treated rats has been used as a selective marker for P-450c activity (30). As shown in Fig. 4, all compounds except DDC caused significant NADPH-dependent inhibition of 7ERFOD activity. Although NADPH dependence is only one criterion for mechanism-based inactivation of P-450, we believe that our data together with the known effects of these compounds on microsomal P-450 implicate P-450c as a target for mechanism-based inactivation by DDC analogues. One analogue, 4-isobutyl-DDC also causes inhibition of 7ERFOD activity when incubated in the absence of NADPH. This NADPH-independent inhibition likely results from the inability of centrifugation to remove residual 4-isobutyl-DDC.

Second, we have employed 7PRFOD activity in microsomes from PB-treated rats as a marker for P-450b function (30). All

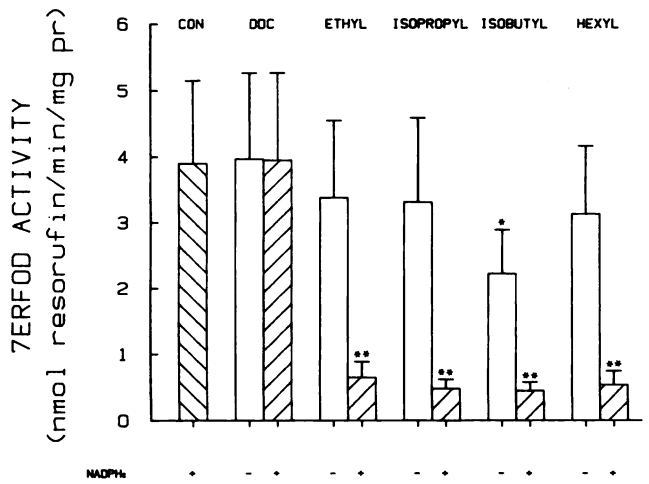

Fig. 4. Effect of DDC analogues on 7-ethoxyresorufin *O*-deethylase activity in microsomes from βNF-treated rats. Microsomes were incubated for 30 min with the respective DDC analogue in the presence (+) or absence (−) of NADPH, reharvested by centrifugation, and then assayed for enzyme activity. Control (*CON*) represents initial incubation in the presence of NADPH alone. Each *bar* represents the mean (± standard deviation) of determinations from four rats. *Significantly different ($p \leq 0.01$) from control; **significantly different ($p \leq 0.01$) from control and incubation in the presence of the respective DDC analogue in the absence of NADPH, as determined by a repeated-measures design one-way analysis of variance and Newman-Kuels test.

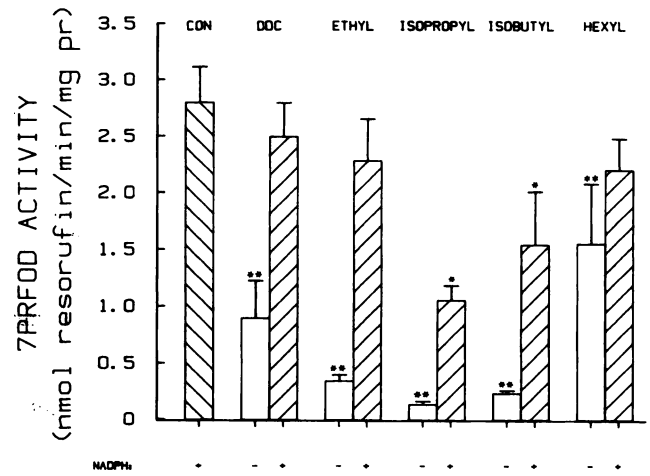

Fig. 5. Effect of DDC analogues on 7PRFOD activity in microsomes from PB-treated rats. Legend as in Fig. 4, except **significantly different ($p \leq 0.01$) from control and incubation in the presence of the respective DDC analogue and NADPH.

compounds, including DDC, caused significant inhibition of this marker activity (Fig. 5). However, in this case, greater inhibition is seen in the absence of NADPH, and inclusion of NADPH in the initial preincubation tends to return activity toward control levels, presumably by allowing the DDC analogues to be metabolized to products that do not interfere with 7PRFOD activity. Such a pattern of inhibition is inconsistent with mechanism-based inactivation of P-450b. The data suggest that DDC analogues are not removed from microsomes from PB-treated rats by centrifugation and that these compounds directly inhibit P-450b function in a nonsuicidal fashion. We have performed kinetic analyses to examine this direct NADPH-independent inhibition of 7PRFOD activity by 4-ethyl-DDC in greater detail. Using various concentrations of the substrate 7-pentoxyresorufin, we have measured activity in the absence and presence of 10^{-8} and 10^{-6} M 4-ethyl-DDC. An Eadie-Hofstee plot (Fig. 6) demonstrates that 4-ethyl-DDC functions as a reversible mixed-type inhibitor, influencing both the apparent K_m and V_{max} for this reaction. The presence of 10^{-8} and 10^{-6} M 4-ethyl-DDC increases the K_m for 7-pentoxyresorufin from 1.50 μM to 2.16 and 3.73 μM, respectively, and decreases the V_{max} from 2.50 nmol/min/mg of protein to 2.12 and 0.85 nmol/min/mg of protein, respectively. A Dixon analysis (data not shown) indicates that 4-ethyl-DDC interacts with isozymes catalyzing 7PRFOD with a K_i of 2.1×10^{-7} M.

Finally, we have used ERND activity in microsomes from DEX-treated rats as a marker for the catalytic function of P-450p-related isozymes (31). In this case, 4-ethyl-DDC, 4-isopropyl-DDC, and 4-isobutyl-DDC caused significant NADPH-dependent inhibition of ERND activity (Fig. 7), which we believe reflects mechanism-based inactivation of P-450p. In addition, 4-isopropyl-DDC caused significant NADPH-independent enzyme inhibition, indicating a strong interaction between this analogue and P-450p. DDC and 4-hexyl-DDC did not effect ERND activity.

Discussion

Several analogues of DDC cause mechanism-based inactivation of P-450, resulting in the formation of an *N*-alkylPP (2–

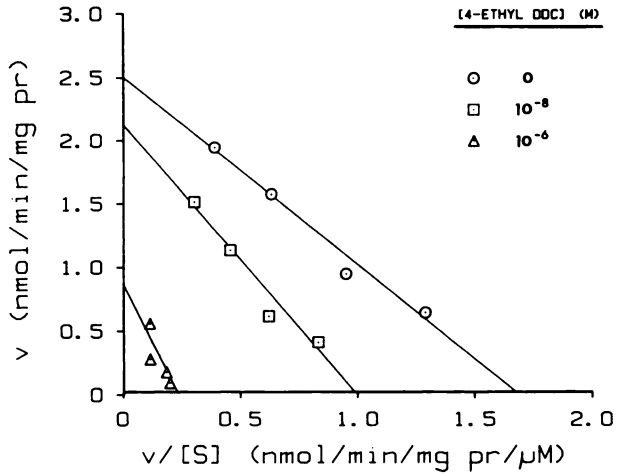

Fig. 6. Eadie-Hofstee plot demonstrating the effect of 4-ethyl-DDC on 7PRFOD activity in microsomes from PB-treated rats. Each *point* represents the mean of three determinations.

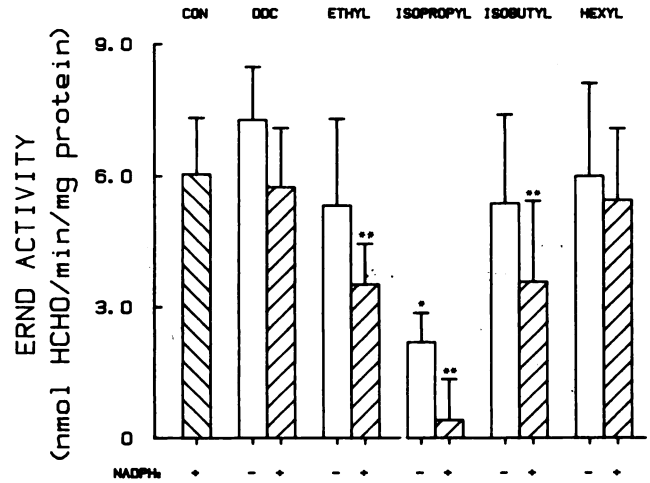

Fig. 7. Effect of DDC analogues on ERND activity in microsomes from DEX-treated rats. Legend as in Fig. 4.

4). With different DDC analogues, the regioisomer composition of the resulting *N*-alkylPP differs and this contributes to the differences in ferrochelatase-lowering potency observed with these compounds (7). It has not been determined whether all four regioisomers of an *N*-alkylPP are formed on the same P-450 enzymic site or whether individual P-450 isozymes selectively direct alkylation to one of the four pyrrole nitrogens of the prosthetic heme. An understanding of this problem will clarify the porphyrinogenic mechanism of the DDC analogues, as well as contribute to our knowledge of the active site characteristics of P-450 isozymes. As a step toward this goal, our objective was to examine the isozyme selectivity of DDC analogues with respect to the major inducible isozymes of P-450.

We measured P-450 loss in microsomes from untreated, PB-treated, βNF-treated, and DEX-treated rats (Fig. 2) to determine whether isozyme-selective patterns of destruction exist for DDC analogues, as was shown for other suicide substrates of P-450 (32). The inability of DDC to cause significant destruction of P-450 in rat liver microsomes, despite its ability to destroy 22% of chick embryo hepatic microsomal P-450 (3), is likely due to differences in the P-450 isozyme patterns between species.

The patterns of P-450 destruction caused by DDC analogues following pretreatment with P-450 inducers reveal that analogues display different P-450 isozyme selectivities. The ethyl and isobutyl analogues appear to inactivate constitutive as well as inducible P-450 isozymes in a relatively nonselective manner. Correia *et al.* (20) have previously shown that 4-ethyl-DDC causes destruction of 40 and 35% of the P-450 in microsomes from untreated and PB-treated rats, respectively. However, this group found that 71% of the P-450 present in microsomes from DEX-treated rats is destroyed by 4-ethyl-DDC, as compared with our finding of 50%. The isopropyl analogue seems to be a very effective inactivator of DEX-inducible isozymes but a relatively poor inactivator of βNF-inducible isozymes, compared with constitutive forms. 4-Hexyl-DDC appears to be a relatively poor inactivator of isozymes induced by PB and DEX, compared with constitutive isozymes.

The second aim of this work was to examine the effect of DDC analogues on the apoprotein moiety of selected P-450 isozymes in a microsomal incubation. It was previously believed that DDC analogues caused heme alkylation, leaving the P-450 apoprotein intact. However, Tephly *et al.* (19) were the first to demonstrate a loss of P-450 c, h, k, and p immunoreactivity following the administration of 4-ethyl-DDC to rats. The mechanism of apoprotein loss is not known but may involve a direct attack by reactive metabolites, the production of heme-derived protein adducts, or catabolic destruction of the apoprotein promoted by loss of the heme prosthetic group. We were interested in determining whether a similar alteration of the apoprotein could be observed in an *in vitro* microsomal preparation. In this work, we report a selective alteration of the immunoreactive protein moiety of P-450c, following an *in vitro* incubation, determined using MAb 1-31-2 (Fig. 3B) and 1-36-1 (Fig. 3C). The Western blot technique employed appears to lack the sensitivity to detect differences in the density of the main P-450c isozyme bands but can clearly demonstrate the formation of lower molecular mass immunoreactive protein bands, which may represent DDC analogue-induced degradative fragments. Although nonspecific proteolysis cannot be definitely ruled out as an explanation for this finding, the fact that these immunoreactive bands of faster mobility are consistently and significantly ($p \leq 0.05$) elevated by DDC analogue treatment suggests that the suicide substrates can promote at least limited protein catabolism *in vitro*. By Western blot analysis, we also demonstrated alteration of the immunoreactive protein of P-450p-related forms, in microsomes from DEX-treated rats, by DDC analogues (Fig. 3F). However, in this case, analysis with MAb 2-13-1 showed that DDC analogues increased the levels of immunoreactive protein bands of molecular mass 80, 94, and 115 kDa. These immunoreactive bands likely represent aggregates of P-450p-related isozymes altered by DDC analogues. The formation of both lower and higher molecular mass immunoreactive protein bands in an *in vitro* microsomal system has been demonstrated previously for the P-450 suicide substrate carbon tetrachloride, using a polyclonal antibody directed against the PB-inducible 54-kDa isozyme of rat liver (8). In this case, both the parent 54-kDa isozyme and the aggregates of >200 kDa had heme covalently bound, whereas the immunoreactive 29-kDa fragment did not. It will be of interest to examine the extent of heme covalent binding to the altered immunoreactive proteins observed in this study.

The significance of the small alterations in immunoreactivity observed in microsomes from βNF-treated and DEX-treated rats must be questioned, in light of the rather dramatic decreases in P-450 c and p catalytic activities caused by DDC analogues. In all cases, the amount of immunoreactive material in the main isozyme band does not change, indicating that only a very small fraction of the protein is altered. Thus, it seems evident that enzyme inactivation is caused by heme destruction, not protein alteration, and that changes in the apoprotein may be a response to enzyme inactivation, which can only occur to a limited extent in a microsomal system. In contrast, suicide substrates of P-450, including AIA and 4-ethyl-DDC, cause loss of the apoprotein of selected P-450 isozymes when administered *in vivo* (19, 20, 33). Thus, it appears that P-450 isozymes altered by suicide substrates *in vitro* escape, for the most part, mechanisms (e.g., proteases) that are operative *in vivo* for the degradation and removal of inactivated enzymes.

Concurrently with our immunochemical studies, we examined the effect of DDC analogues on enzymic functions selective for P-450 c, b, and p. With the exception of DDC, which was inactive, all compounds caused 83–88% inhibition of 7ERFOD activity in microsomes from βNF-treated rats in an NADPH-dependent manner. Thus, P-450c is a target for mechanism-based inactivation by DDC analogues. In rats treated with βNF, P-450c is believed to represent nearly 45% of the total P-450, as compared with 2% in the uninduced state (16). However, induction with βNF does not increase the percentage of P-450 susceptible to destruction by DDC analogues (Fig. 2). This apparently contradictory finding can be better understood by considering the effect that βNF pretreatment has on other P-450 isozymes. Treatment with βNF is known to decrease the levels of P-450 h, k, and p (16), isozymes known to be important targets for 4-ethyl-DDC (20). Thus, in microsomes from βNF-treated rats, P-450c is likely a major target for inactivation by DDC analogues.

In microsomes from PB-treated rats, DDC analogues did not cause mechanism-based loss of 7PRFOD activity (Fig. 5), a catalytic marker for P-450b. While these studies were in progress, it was reported that, in a purified reconstituted system, P-450b did not metabolize 4-ethyl-DDC and was not a target

for 4-ethyl-DDC-mediated inactivation (34). All analogues appeared to be tightly associated with this isozyme, however, and showed NADPH-independent inhibition of 7PRFOD activity. Two analogues, 4-isopropyl-DDC and 4-isobutyl-DDC, were particularly active in this regard. A representative analogue, 4-ethyl-DDC, was shown to function as a reversible mixed-type inhibitor of 7PRFOD activity (Fig. 6). Thus, this analogue interferes both with binding of the substrate 7-pentoxyresorufin and with the catalytic competence of the enzyme-substrate complex. Thus, it appears that P-450b is not a target for mechanism-based inactivation by DDC analogues and, hence, the ability of DDC analogues to cause P-450 destruction in microsomes from PB-treated rats must reflect the destruction of other PB-inducible isozymes, most likely P-450 k and p, which together may account for 38% of the total P-450 following PB induction (16). Another potential target, P-450h, accounts for nearly 11% of the P-450 in microsomes from PB-treated rats, compared with 54% in the uninduced state (16).

Analogues differed with respect to their ability to reduce P-450p catalytic activity (Fig. 7). As anticipated, DDC itself was inactive, as was the 4-hexyl analogue. The inability of 4-hexyl-DDC to inactivate P-450p likely contributes to the dramatic decrease in P-450 destruction caused by this analogue in microsomes from PB-treated and DEX-treated rats (Fig. 2). Both 4-ethyl-DDC and 4-isobutyl-DDC significantly reduce ERND activity, and yet induction with DEX does not significantly increase the extent of P-450 destruction caused by these analogues (Fig. 2). Similar to the case of βNF induction, pretreatment of rats with PCN significantly suppresses levels of P-450 h and k (16), potential targets for inactivation. In microsomes from PCN-treated rats, P-450p-related forms are believed to represent 58% of the total P-450 (16) and, thus, P-450p-related isozymes are likely the major targets for inactivation by these compounds in our microsomes from DEX-treated rats. The most extensive inactivation of ERND activity was shown by 4-isopropyl-DDC, a finding consistent with the large increase in P-450 destruction caused by this analogue following DEX induction. The fact that 4-isopropyl-DDC does not form an *N*-alkylPP is interesting, in light of the fact that P-450p, an isozyme that seems to be a key target for this analogue, is not believed to form an *N*-alkylPP during its interaction with 4-ethyl-DDC (20).

These data demonstrate that DDC analogues interact with the major βNF-inducible isozyme, P-450c, and the major DEX-inducible isozyme, P-450p, to cause loss of catalytic activity and limited alteration of the apoprotein *in vitro*. The elucidation of the P-450 isozyme selectivity of DDC analogues will contribute to our understanding of the differences in porphyrinogenicity of these compounds. It is of interest to determine whether the interaction of DDC analogues with specific P-450 isozymes results in the formation of heme-protein adducts and/or unique regioisomers of *N*-alkylPP. This information is critical in determining whether the P-450 isozyme or the DDC analogue itself plays a role in directing regioselective alkylation of the prosthetic heme. Such studies will yield information concerning the active site environment of P-450 isozymes.

Acknowledgments

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